Aus dem Institut für Veterinär-Physiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

# Influence of magnesium on endocrine regulation and energy metabolism in bovine adipocytes

**Inaugural-Dissertation**
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
**Sandra Karolina Becker, geborene Jurek**
Tierärztin aus Berlin

Berlin 2022
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*Dla Mamy i Taty*

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## List of abbreviations

| | |
|---|---|
| 6-NBDG | 6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose |
| ACC | Acetyl-CoA carboxylase |
| AKT | AKT Serine/threonine kinase 1 |
| AOX | Acyl-CoA oxidase |
| ASC | Adipose-derived stem cells |
| ATGL | Adipose triglyceride lipase |
| ATP | Adenosine triphosphate |
| BAT | Brown adipose tissue |
| BHB | β-Hydroxybutyrate |
| BSL | Bovine serum lipids |
| cAMP | Cyclic adenosine monophosphate |
| CD105 | Endoglin (ENG) |
| CD73 | Ecto-5'-nucleotidase (NT5E) |
| CD90 | Thy-1 Cell surface antigen (THY1) |
| CoA | Coenzyme A |
| COX | Cyclooxygenase |
| DHA | Docosahexaenoic acid |
| DLK1 | Delta like non-canonical notch ligand 1 |
| DNA | Deoxyribonucleic acid |
| EGF | Epidermal growth factor |
| ELAM1 | Endothelial leukocyte adhesion molecule |
| EPA | Eicosapentaenoic acid |
| FA | Fatty acids |
| FABP | Fatty acid binding protein |
| FAS | Fatty acid synthetase |
| FATP | Fatty acid transport protein |
| FBS | Fetal bovine serum |
| G6PD | Glucose 6-phosphate dehydrogenase |
| GH | Growth hormone |
| GLUT | Glucose transporter |
| GPDH | Glycerol-3-phosphate dehydrogenase |
| HSL | Hormone-sensitive lipase |
| IBMX | 3-Isobutyl-1-methylxanthine |
| IKKβ | Inhibitor of the NF-κB kinase |
| IL | Interleukin |
| IRS | Insulin receptor substrate |
| JNK | c-Jun N-terminal kinase |
| LDL | Low-density lipoproteins |
| LM | Lipid mediators |

| | |
|---|---|
| LPL | Lipoprotein lipase |
| LPS | Lipopolysaccharide |
| MagT1 | Magnesium transporter 1 |
| MGL | Monoacylglycerol lipase |
| mRNA | Messenger ribonucleic acid |
| MSC | Mesenchymal stem cell |
| mTORC2 | Mammalian target of rapamycin complex 2 |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| NEB | Negative energy balance |
| NEFA | Non-esterified fatty acids |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NIPA1 | NIPA Magnesium transporter 1 |
| PDE-3B | Phosphodiesterase 3B |
| PDK1 | Phosphoinositide-dependent protein kinase-1 |
| $PGE_2$ | Prostaglandin E2 |
| PI3K | Phosphatidylinositol 3-kinase |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PPAR | Proliferator-activated receptor |
| PTH | Parathormone |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SCFA | Short chain fatty acids |
| SLC41 | Solute carrier family 41 |
| SREBP | Sterol regulatory element-binding protein |
| TAG | Triacylglycerols |
| TAL | Thick ascending limb of Henle |
| TCA | Tricarboxylic acid cycle |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TRPM | Transient receptor potential cation channel subfamily M |
| $TXA_2$ | Thromboxane $A_2$ |
| VLDL | Very low-density lipoproteins |
| WAT | White adipose tissue |

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# Chapter 1: Introduction

During the transition period, namely three weeks before and three weeks after calving, the physiological situation of dairy cows changes dramatically. The requirement of energy increases enormously because of rapid fetal development and the onset of lactation (Contreras and Sordillo 2011). The intake of feed dry matter lags behind the demand of energy and nutrients, e.g., glucose. This imbalance between energy intake and energy demand leads to a negative energy balance (NEB) (Wankhade et al. 2017), which can only be compensated by the mobilization of body fat. Such lipolysis, i.e., the release of fatty acids (FA) from triacylglycerols (TAG), leads to a reduction of the size of adipose tissue, together with weight loss, fatty liver, an increased inflammatory response, and a reduction of the secretion of many hormones, e.g., adipokines (Contreras et al. 2017b). Furthermore, this phase is characterized by a reduced insulin sensitivity of many tissues, e.g., muscles, liver, and adipose tissue. By reducing glucose uptake in insulin-dependent tissues, more glucose is available for insulin-independent tissues, such as the mammary gland (Aschenbach et al. 2010). During this phase of glucose shortage, ketone bodies, namely acetoacetate, β-hydroxybutyrate (BHB), and acetone, serve as the main energy source for peripheral tissues and are synthesized in the liver from FA, which are the products of lipolysis (Laffel 1999). These adaptations are necessary and physiological. However, if a prolonged NEB with exaggerated and excessive lipolysis occurs, metabolic disfunctions occur and are made responsible for many diseases of dairy cows such as ketosis, milk fever, mastitis, and metritis (Esposito et al. 2014). Thus, adipose tissue plays a crucial role in the energy state, lipid and carbohydrate metabolism, insulin sensitivity, and consequently, the health status of dairy cows. Magnesium, as the second most prevalent intracellular cation, is an essential cofactor in many enzymatic pathways (Jahnen-Dechent and Ketteler 2012). Magnesium homeostasis is tightly regulated by various factors, the most important being insulin. In turn, insulin secretion and signaling are affected by magnesium in its role as a second messenger (Paolisso et al. 1990). As is known for diabetes type 2 in humans, an over-supplementation of magnesium can improve the insulin sensitivity of adipocytes and therefore restrict excessive lipolysis with all its negative effects on health (Verma and Garg 2017). The aim of this study was to study the influence and interaction of magnesium and insulin in cultured bovine adipocytes in order to possibly unravel insulin-mediated anti-lipolytic signaling pathways and their responsiveness to magnesium supplementation in cattle. A better understanding of these metabolic interactions in bovine adipocytes might aid the discovery of new therapeutic strategies for the treatment of diseases connected to the status of dairy cows during the transition period.

# Chapter 2: Literature review

## 2.1 Transition period of dairy cows

Milk consumption in the developed world has risen within the last few decades as an integral part of the diet in western countries (Pereira 2014; Wiley 2014). The dairy industry has grown substantially (Arvanitoyannis and Kassaveti 2008) and selection for high-milk-yielding cows with continuously rising production of milk per animal and per day has become possible by achievements in veterinary science and breeding (Kushwaha et al. 2011). Milk yields of more than 10,000 liters per lactation period are not uncommon but are not without serious consequences for the health and welfare of dairy cows (Baumgard et al. 2017). The transition period of dairy cows occurs when the body changes from the pregnant non-lactating state to the postnatal lactating stage (Contreras et al. 2017b) and is defined as the time of approximately three weeks before and three weeks after calving (Contreras and Sordillo 2011). During this phase, the dairy cow goes through the most critical period in its production cycle and life (Contreras and Sordillo 2011). Typical hormonal changes, e.g., increasing amounts of prolactin and growth hormone (GH) and the reduction of progesterone, initiate the transition period (Contreras et al. 2017b), which represents a time of catabolism for the animal because of rapid fetal growth, metabolic adaptation in preparation for upcoming lactogenesis, lactation, and the adaptation of ruminal metabolism to a changing diet (Promkot et al. 2011). Dry matter intake lags behind the tremendous increase in energy demand inevitably leading to a negative energy balance (NEB). The physiology of nearly all peripartum dairy cattle becomes problematic because of, for example, insulin resistance, NEB, weight loss and lipolysis, hypocalcemia, and immunological disfunctions (Leblanc 2010). For the production of milk during lactation, glucose is extensively depleted from the blood by the mammary gland to produce lactose and milk proteins (Bell 1995). In order to ensure the supply of glucose for milk production, insulin-dependent tissues, such as skeletal muscles, adipose tissue, and liver show a reduced insulin sensitivity in order to raise the availability of glucose to insulin-independent tissues such as the mammary gland and therefore for milk production (Aschenbach et al. 2010). This insulin insensitivity during the transition period is a physiologically reasonable and necessary process. During the period of glucose shortage, the dairy cow needs an alternative energy source. Therefore, ketone bodies, namely acetoacetate, β-hydroxybutyrate (BHB), and acetone, serve as energy providers for peripheral tissues. Ketone bodies are synthesized in the liver from FA mobilized from adipose tissue (Laffel 1999). In order to cover the need of this energy metabolite, FA from triacylglycerols (TAG) are metabolized from adipose tissue, thereby explaining the observed reduction in adipose tissue size, the increase in lipolysis, and weight loss (Contreras et al. 2017b). When the energy status is balanced, ketones are present in small amounts in the systemic circulation as a result of

their synthesis in the rumen epithelium (Enjalbert et al. 2001). During the transition period with high energy demand, the concentration of ketone bodies increases rapidly as they are the most important source of energy for the brain in periods with insufficient energy intake (Laffel 1999). An exaggeration of this physiological adaptation predisposes cows to metabolic and inflammatory diseases (Contreras et al. 2017b), and biological processes in adipocytes, liver, gut, and mammary gland become unbalanced (Drackley 1999).

### 2.1.1 Metabolic diseases of high-milk-producing cows

Metabolic disorders and associated inflammatory diseases are a crucial problem during the transition period in cows. The health and performance of the high-yielding cow are impaired and the profitability of the dairy cow consequently drops dramatically. Steeneveld et al. have described losses, on average, of 709 € for a clinical ketosis case and, on average, of 150 € for a subclinical ketosis case with regard to costs of treatment and diagnosis and those related to losses in milk production (Steeneveld et al. 2020). All in all, economic disadvantages incur, and most importantly, an ethical problem arises because of culling interventions attributable to loss of performance and disease.

The most common diseases, such as ketosis and fatty liver, infectious mastitis, displacement of the abomasum, or retained placenta, occur within the first two weeks of lactation (Goff and Horst 1997; Van Saun and Sniffen 2014), with 30% to 50% of dairy cows possibly being affected by some of these diseases (Leblanc 2010; Sundrum 2015). As described above, the energy demand rises dramatically, and the body needs to compensate for this lack of energy. The dry matter intake lags behind the nutrient requirements, and the cow enters a state of energy shortage, namely NEB (Fenwick et al. 2008), resulting in a glucose deficiency with low glucose blood concentrations and the mobilization of body reserves in order to deliver additional energy (Sundrum 2015). The interplay of glucose deficiency and the mobilization of body reserves, especially from adipose tissue, often marks the beginning of a period of suffering in dairy cows.

#### *2.1.1.1 Fatty liver and ketosis*

Ketosis and the closely associated so-called fatty liver are among the major diseases of dairy cattle in the world (Shaw 1956), Approximately 50% of dairy cows suffer from ketosis or concomitant diseases around the time of calving (Bobe et al. 2004; Leblanc 2010). As described above, these ailments begin with NEB. The metabolic change-over in the transition period leads to NEB because of the drastic rising demand of energy for the maintenance of late gestation, lactogenesis and galactopoesis, accompanied by a decreased dry matter intake during the dry period. This situation goes from bad to worse after the birth: the energy demand for lactation is higher than the capacity of ingestion (Gordon et al. 2013).

Furthermore, the need of glucose for milk production rises, so that the concentration of glucose in the blood is too low. The glucose absorption in the gut of cattle is minimal, and hence, the glucose need has to be covered by gluconeogenesis in the liver (Gordon et al. 2013). The NEB, together with the resulting low blood glucose and low insulin concentration, leads to a mobilization of FA from fat stores (Van Der Drift 2013). Adipocytes, as a main energy storage, contain TAG that are synthesized from FA during periods with a positive energy balance by the process of lipogenesis (Contreras et al. 2017b). In the case of NEB, the fat metabolism switches from an anabolic to a catabolic metabolism by activating lipases which effect a release of glycerol and free FA, also known as non-esterified fatty acids (NEFA), from TAG molecules. Glycerol and NEFA constitute a major source of energy during the time of NEB (Tessari et al. 2020). The transport of NEFA in the blood is ensured by binding to albumin; nevertheless, a small amount is transported as unbound monomers and is available for oxidation in many tissues (Contreras et al. 2010). Most of the NEFA are transported to the liver, which has a key role in lipid metabolism. They can be transported into the liver by transporters, e.g., fatty acid transport protein (FATP), or via diffusion (Nguyen et al. 2008). Subsequently, they are used for energy production via the β-oxidation pathway where they are broken down to acetyl-CoA for the tricarboxylic acid cycle (TCA or Krebs cycle), or the NEFA can be re-esterified into TAG and transported as very low-density lipoproteins (VLDL) in the blood circulation (Rui 2014). During NEB, the liver might be overloaded with a large amount of NEFA. The oxidation of FA, hydrolysis, and the export of TAG into the circulation via VLDL can over-challenge the capacity and function of the liver, resulting in an accumulation of TAG in the liver tissue and hence in a hepatic lipidosis (Mohamed et al. 2004). Another crucial factor contributing to the accumulation of TAG is that ruminants have low synthesis and export rates for VLDL in comparison with other animal species (Grummer 1993; Drackley 1999). Overall, fatty liver occurs when the uptake of NEFA exceeds their metabolization and the export of lipids. The infiltration of lipid droplets is associated with a limitation of the function of hepatocytes because of a loss of cellular integrity and inflammation, possibly leading to necrosis. The cow therefore experiences an impairment in its health status, in its productivity and milk production, in its reproduction, and finally in its life span (Bobe et al. 2004). Its health status deteriorates because of far-reaching consequences to its whole body because of the limitation of metabolic processes such as carbohydrate metabolism with a lower gluconeogenesis rate, a lower ability to store glycogen, and therefore a higher production of ketone bodies. Furthermore, restrictions in lipid and protein metabolism exacerbate the situation: the production of VLDL is impaired and the detoxification of urea is reduced because of a worsening of the loss of hepatocyte function (Katoh 2002; Bobe et al. 2004).

Ketosis occurs collateral to fatty liver in dairy cows. Ketogenesis, the process of the production production of ketone bodies, namely acetoacetate, BHB, and acetone. Ketogenesis increases with high blood NEFA concentrations and accordingly enhanced NEFA uptake into the liver (Bobe et al. 2004; Nguyen et al. 2008). Furthermore, the production of ketone bodies rises during high concentrations of acetyl-CoA because of the upregulation of gluconeogenesis and therefore the removal of oxaloacetate to the TCA cycle (White 2015; Tessari et al. 2020). In times of NEB, this synthesis of ketone bodies allows the metabolization of more energy from FA (with the same number of ATP molecules), in a water-soluble form as a vital fuel for peripheral tissues in times of glucose shortage (Laffel 1999; Nguyen et al. 2008). However, the overstressing of this physiological adaptation with an increased concentration of NEFA and ketone bodies is toxic and influences metabolic functions (Bobe et al. 2004).

The ketosis of cows can be differentiated into a subclinical and clinical ketosis and is linked to the concentration of BHB in the blood (Suthar et al. 2013). Subclinical ketosis is defined by a BHB concentration of 1.4 mmol/L without clinical signs, whereas cows with clinical signs and a BHB concentration of more than 3 mmol/L have clinical ketosis (Mcart et al. 2012; Suthar et al. 2013).

Animals with high concentrations of ketone bodies in the blood around the time of calving have a higher risk of suffering from diseases in comparison with animals having low plasma concentrations (Itle et al. 2015). The vicious circle is exacerbated by high concentrations of BHB that decrease the rate of gluconeogenesis, β-oxidation, and the function of the TCA cycle (Bobe et al. 2004). Taken together, prolonged times of NEB and strong increases in NEFA and ketone bodies have negative effects on the health of the animal and, therefore, these two metabolites can be used as markers for metabolic disorders during the transition period (Tessari et al. 2020).

#### *2.1.1.2 Link between metabolic and inflammatory diseases*

The metabolic changes that occur in the transition period have an impact on many metabolic pathways and on the immune response and therefore affect the health of dairy cows (Sordillo and Raphael 2013). Immune responses of dairy cows are directly influenced by ketone bodies (Suthar et al. 2013). Almost half of dairy cows with periparturient diseases have BHB concentrations above the threshold and show reduced performance in production, health, and reproduction (Mcart et al. 2013; Leblanc 2020). The consequence is immunosuppression, which is not completely understood, although fatty liver has been suggested to decrease the immune response by influencing the phenotypic and functional properties of neutrophils (Drackley et al. 2005).

The main diseases observed in dairy cows are milk fever, displaced abomasum, retained placenta, metritis, mastitis, lameness, and pneumonia (Vergara et al. 2014). Milk fever is

explained by an enormous drop of calcium in the blood serum because of the onset of lactation. Furthermore, hypocalcemia causes changes in smooth muscles cells leading to displacement of the abomasum (Drackley et al. 2005) and to higher cortisol secretion with a potential influence on retaining placenta (Goff and Horst 1997). Inflammation and infectious diseases, such as metritis, mastitis, lameness, or pneumonia, have their causes in the insufficient function of neutrophils (Leblanc 2020). Fat mobilization and, therefore, an increase of NEFA and BHB together with oxidative stress, reduced glucose and calcium availability, and finally social stressors influence the functional capacity of neutrophils by reducing the expression of important surface molecules and thereby also influence the cytotoxicity of blood neutrophils (Drackley et al. 2005; Leblanc 2020).

The interaction of metabolic dysfunction (namely fat metabolism), immune function, and inflammation is known in humans and dairy cows. The secretion of tumor necrosis factor α (TNFα), interleukin 1 (IL-1) and interleukin 6 (IL-6), which have their origin in the high amounts of NEFA from lipolysis that activate the toll-like receptor 4 (TLR4), increase inflammation and insulin resistance (Sordillo and Raphael 2013; Leblanc 2020) thereby exacerbating the vicious circle of derailed metabolism during the transition period.

#### *2.1.1.3 Glucose metabolism and insulin resistance*

From late pregnancy until the beginning of lactation, the glucose requirement in cows quadruples (Zachut et al. 2013). The gravid uterus and lactogenesis cause an extreme consumption of glucose, the precursor of lactose. Endogenous glucose production rises, and the peripheral glucose uptake decreases to ensure the glucose supply to the mammary gland (Aschenbach et al. 2010; Weber et al. 2013). Because of low resorption of glucose from the intestine, most of the glucose in ruminants originates from gluconeogenesis in the liver and kidney. The most important precursor molecule for gluconeogenesis is propionate, which is synthesized via microbial carbohydrate fermentation in the forestomach (De Koster and Opsomer 2013). Furthermore, the liver is capable of synthesizing glycogen during periods of sufficient energy supply. In times of NEB, glycogen can be mobilized in order to raise the blood glucose level directly by glycogenolysis (De Koster and Opsomer 2013). To support this, glycogenolysis is increased in skeletal muscle. Because of the absence of glucose-6-phosphatase (converting glucose-6-phosphate into glucose), muscle glucose oxidation results in lactate, which can be used in the liver for gluconeogenesis, which influences the glucose blood concentration indirectly (De Koster and Opsomer 2013). Furthermore, muscle and adipocyte breakdown can deliver metabolites, namely amino acids and glycerol, for the synthesis of glucose in the liver (Bell and Bauman 1997). Mobilized glucose is taken up by a group of glucose transporters from the GLUT family. GLUT isoform 1 (GLUT1) is a ubiquitous transporter and is responsible for the basal glucose uptake (Bell and Bauman 1997). This

transporter is upregulated in the mammary gland during lactation and is insulin-independent (De Koster and Opsomer 2013). GLUT4, the only insulin-stimulated glucose transporter, is expressed in several tissues, e.g., adipose tissue and muscles, but not in the mammary gland (O'boyle et al. 2012). Upon insulin stimulation, GLUT4 is translocated from intracellular stores to the plasma membrane in order to reduce blood glucose concentrations (De Koster and Opsomer 2013). The insulin receptor consists of an α and β subunit (Khan and Pessin 2002), where the binding of insulin leads to the phosphorylation of the β subunit itself, activating the insulin receptor, which in turn results in the phosphorylation of insulin receptor substrates 1 and 2 (IRS1/2) (Myers and White 1996) and the subsequent activation of PI3K (phosphatidylinositol 3-kinases) and PDK1 (phosphoinositide-dependent protein kinase-1) followed by the activation of Akt (protein kinase B (PKB)) (Kumar et al. 2012). The PI3K-AKT/PKB signaling pathway then activates further metabolic processes, such as glucose transport, glycogen synthesis, and lipogenesis, whereas gluconeogenesis and lipolysis are inhibited (Jung and Choi 2014).

During late pregnancy and early lactation, glucose metabolism shows homeorhetic changes. The expression of the glucose transporter in skeletal muscle and adipocytes is decreased and thus glucose consumption is reduced, sparing glucose for milk production (Bell and Bauman 1997). In order to support this adaptation, cattle also show insulin resistance in peripheral tissues similar to type 2 diabetes in humans (De Koster and Opsomer 2013). Insulin resistance is defined as a condition in which sufficient insulin is present, but the responsiveness of the tissue to insulin is reduced (Wilcox 2005). The homeorhetic mechanism of insulin resistance is well known not only in dairy cattle, but also in other mammals including humans during the period of late pregnancy and after birth (De Koster and Opsomer 2013). However, genetic breeding for the trait "milk yield" shows an exaggeration of insulin resistance and other metabolic changes with far-reaching consequences (Kushwaha et al. 2011). Insulin is well established as having an influence on many metabolic pathways in the body. This includes its influence on the metabolism of glucose, protein and fat (Kalhan 2009) and therefore the whole energy metabolic system of the body. Adipocytes respond strongly to insulin not only by increasing glucose uptake, but also by taking up NEFA, thus increasing lipogenesis and inhibiting lipolysis (Contreras et al. 2017b). Furthermore, insulin plays an extraordinary role in cell growth and differentiation (Petersen and Shulman 2018). If insulin resistance interacts with other metabolic dysfunctions, the consequences for animal health are far-reaching. The lack of an insulin signal in some tissues and the associated intracellular glucose deficiency combined with NEB exacerbate the signal of energy deficiency. Therefore, as has been known for some years, the diseases of dairy cattle are caused not only by insulin resistance, but by an interaction of several factors, e.g., increased fat mobilization to compensate for this energy deficiency. The role of adipose tissue in the insulin sensitivity of the body has been revealed

to be essential (De Koster and Opsomer 2013; Contreras et al. 2017b). Thus, special attention must be paid to the fat metabolism of high-yielding cattle because, as mentioned above, the negative energy balance causes massive metabolic changes in adipose tissue that have far-reaching consequences for insulin sensitivity and, hence, for the entire body.

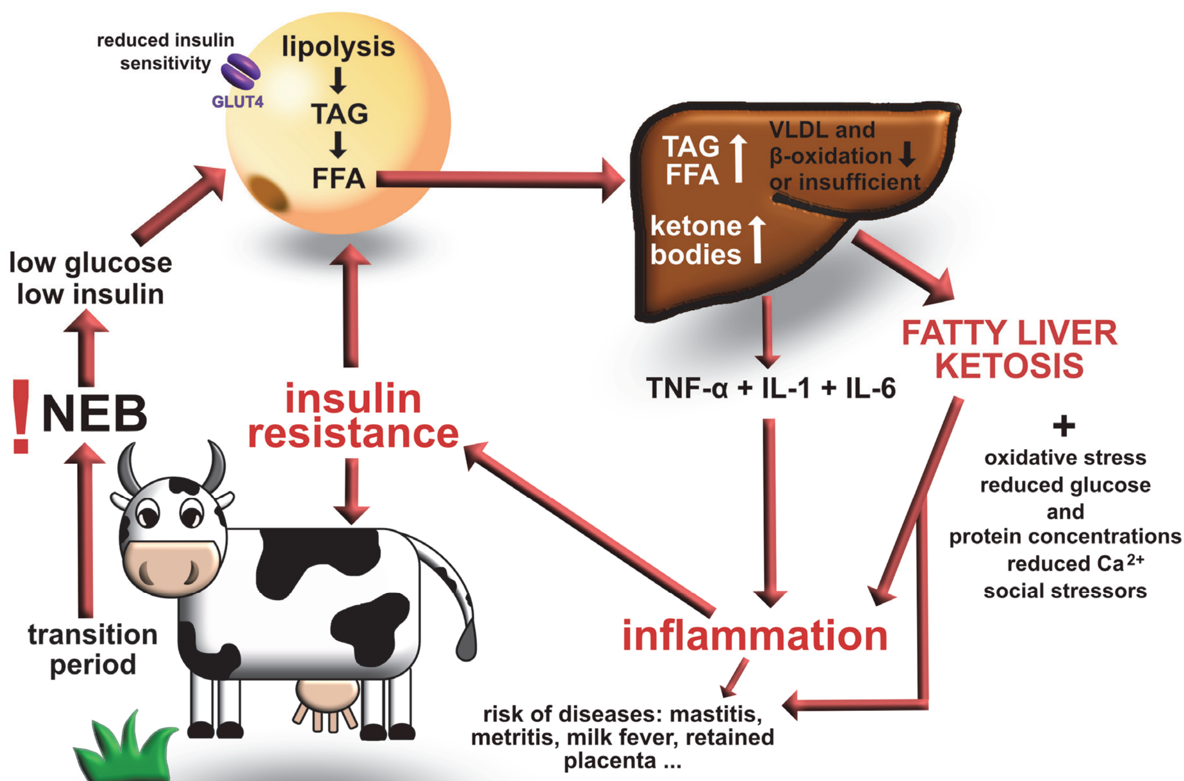


**Figure 1: Schematic illustration of the vicious circle of cows during the transition period.** During the transition period cows suffer from a negative energy balance (NEB) due to the high energy requirements for fetal growth and milk production. Blood concentration of glucose and insulin are low in this time. In addition, there is a reduced insulin sensitivity of the adipocytes and other tissues in order to provide the insulin-independent mammary gland with glucose. The consequences are increased lipolysis in adipose tissue, in which NEFA are released from TAG in order to cover the energy demand of the body. If this physiological mechanism overreacts, a high amount of NEFA enter the liver and exceed the metabolic and transport capacity of the liver. This leads to fatty liver syndrome, release of inflammatory mediators (TNF-α, IL-1 and IL-6), and ketosis. The result is widespread inflammation, which increases the risk of possible diseases and forces insulin resistance. A vicious circle that is difficult to escape for the animals (own graphic created for this work 2021).

## 2.2 Adipose tissue

After years of being ignored, adipose tissue has received increasing scientific attention in recent years. It not only represents the most important energy reserve of the body, but is also a complex and highly metabolically active organ with many endocrine functions (Wertheimer and Shapiro 1948; Kershaw and Flier 2004). Fatty tissue can be divided into white (WAT) and

brown adipose (BAT) tissue (Ailhaud et al. 1992). WAT can be further classified into subcutaneous adipose tissue and adipose tissue that coats the inner organs and is thus called visceral adipose tissue (Choe et al. 2016). For the sake of simplicity and because of its greater relevance here, this work focuses on WAT. Adipose tissue is largely composed of fat cells and precursor cells in various differentiation stages (mesenchymal stem cells and preadipocytes), although endothelial cells, nerve tissue, immune cells, and tissue matrix, especially fibroblasts, are also present (Ailhaud et al. 1992; Kershaw and Flier 2004). An adult fat cell consists of a large fat vacuole, containing TAG, which pushes the cell organelles and the cell nucleus to the edge of the cell (Poulos et al. 2010). Adipocytes have their origin in mesenchymal stem cells (MSC), which are multipotent progenitor cells that can differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes (Caplan 1991; Minguell et al. 2001). The proliferation and determination of MSC in adipose tissue is followed by the formation of adipoblasts and hence to the production of preadipocytes (Ailhaud et al. 1992). Certain triggers cause the preadipocytes to change their shape and to start dividing. Subsequently, specific genes are activated that allow the synthesis and storage of FA (TAG) (Abdel-Sattar et al. 2014). Because not all the cells in adipose tissue differentiate, adipose tissue contains cells in many differentiation stages, thereby simplifying the collection of stem cells for research purposes, for example, when taking biopsies (Poulos et al. 2010). Thus, adipose tissue is a constantly changing tissue with differentiation and the accumulation and degradation of fat taking place continuously. Fat cells can build up and store fats throughout their life when energy supplies are sufficient in a process called lipogenesis. During times of poor energy supply and NEB, TAG can be mobilized providing energy for the body in a process called lipolysis (Poulos et al. 2010; Contreras et al. 2017b). Therefore, fat cells are not only receivers of energy and information from the body, but more importantly, also act as an energy warehouse transmitting a wide range of information that influences many processes in the body.

### 2.2.1 Lipid metabolism and lipogenesis

Cells of the adipose tissue, the major energy reservoir in the body, undergo major changes during their lifetime. They can store energy, differentiate into additional precursor cells when even larger energy stores are required, and release large amounts of fuel for other cells when energy is scarce (Choe et al. 2016). Adipose tissue is capable of growth; its cells can change their size (hypertrophy) or number (hyperplasia) or both (Laliotis et al. 2010). The fat metabolism cycle consists of fat formation in the adipose tissue, the synthesis and storage of FA, the uptake of FA from the bloodstream, and finally, the release of fats by the degradation of stores and their release into the bloodstream (Pothoven et al. 1975). This physiological adaptation of energy storage and release guarantees the survival of the organism and requires some coordination and regulation.

At times when energy supply is secured, i.e., under condition of positive energy balance, fat is built up and stored in adipocytes by lipogenesis (Contreras et al. 2017b). In ruminants, the main site of FA production is the adipose tissue, whereas in non-ruminants, lipogenesis occurs mainly in the liver (Dodson et al. 2010). The main substrate used for the *de novo* synthesis of FA in subcutaneous adipose tissue in ruminants is acetate and not glucose as in non-ruminants (Dodson et al. 2010). One possible reason for this could be that glucose is hardly absorbed from the intestine because of fermentation by bacteria and must therefore first be synthesized during gluconeogenesis. Thus, the use of glucose for FA synthesis would be much more elaborate than the use of acetate as a precursor molecule in ruminants (Vernon 1980). FA are not resorbed directly in the small intestine of ruminants but are fermented by microbes within the rumen into SCFA(Dodson et al. 2010). These SCFA are absorbed across the ruminal epithelium. The most prevalent SCFA are acetate, propionate, and butyrate (Bergman 1990). Propionate takes part in gluconeogenesis in the liver. Butyrate is metabolized to a high degree into ketone bodies. Butyrate, ketone bodies can be used for lipogenesis together with acetate, the main substrate for the *de novo* synthesis of FA, after conversion into acetyl-CoA by acetyl-CoA synthetase in adipocytes (Burns 2011; Ladeira et al. 2016). Acetyl-CoA is further converted by carboxylation into malonyl-CoA via acetyl-CoA carboxylase (ACC), followed by the formation of long-chain FA by fatty acid synthetase (FAS), while more acetyl CoA molecules are formed into malonyl-CoA molecules (Ladeira et al. 2016). In the cytoplasm of adipocytes, three molecules of FA and one molecule of glycerol are esterified, resulting in a molecule of TAG (Contreras et al. 2018). Reducing equivalents, namely NADPH, are needed to ensure that the novo fatty acid synthesis takes place (Laliotis et al. 2010). NADPH is provided by the pentose phosphate pathway and, especially in ruminants, by the transformation of citrate in the TCA cycle (Ladeira et al. 2016). The final product of FA synthesis is palmitate, which is used as the main substrate by enzymes such as reductases and elongases for the additional synthesis of FA (Laliotis et al. 2010). In times of good energy supply, these FA are stored in the form of TAG in adipocytes (Contreras et al. 2017b). Adipocytes are the cells that respond most strongly to insulin by increasing glucose and NEFA uptake from the circulation, increasing lipogenesis, and inhibiting lipolysis (Contreras et al. 2017b). As soon as insulin binds to adipocytes, insulin receptor tyrosine kinase is activated, resulting in phosphorylation of insulin receptor substrates 1 and 2 (IRS-1/IRS-2). This leads to the activation of a cascade in which phosphatidylinositol 3-kinase (PI3K) is activated first, followed by protein kinase B (AKT), which in turn results in GLUT-4 being incorporated into the cell membrane and glucose being transported into the cell (Contreras et al. 2017b). In this process, lipolysis is inhibited by insulin by the activation of AKT and the concomitant inhibition of PKA (Contreras et al. 2017b). At the same time, lipogenesis is increased by the activation of the transcription factor SREBP1 through AKT (Cignarelli et al. 2019).

Lipogenesis takes place in a coordinated manner (Laliotis et al. 2010). The most important transcription factors in lipid metabolism are the sterol regulatory element-binding proteins (SREBP), which mediate the expression of the lipogenic genes (Laliotis et al. 2010), and peroxisome proliferator-activated receptor (PPAR), which regulates the storage of FA and adipogenesis (Ladeira et al. 2016). SREBP are divided into three types: SREBP-1a, SREBP-1c and SREBP-2 (Eberle et al. 2004). SREBP-2 regulates the genes of cholesterol metabolism, whereas SREBP-1 is necessary for the regulation of the lipogenic genes and is activated by insulin and glucose (Laliotis et al. 2010). This transcription factor regulates the lipogenic genes, namely acetyl-CoA carboxylase (ACC), fatty acid synthetase (FAS), and glucose 6-phosphate dehydrogenase (G6PD) (Laliotis et al. 2010).

PPAR are divided into three types, namely PPAR alpha (PPARα), beta (PPARβ), and gamma (PPARγ), with overlapping functions and expression patterns (Martin 2010), modulating the expression of genes involved in lipid and glucose metabolism and inflammatory pathways (Busato and Bionaz 2020). PPARγ, which is predominantly expressed in adipocytes, regulates adipogenesis and FA storage by activating the genes for lipid metabolism, e.g., fatty acid binding protein 4 (FABP4), which transports FA into cells (Ladeira et al. 2016), or acyl-CoA oxidase (AOX), which is the key and rate-limiting enzyme of β-oxidation (Keller et al. 1993; Hihi et al. 2002). Furthermore, PPARγ is needed for the induction of preadipocyte differentiation and their conversion to adipocytes (Jurek et al. 2020), for the remodeling of adipocytes, and for adipokine secretion (Mann et al. 2016). This transcription factor is activated by a range of FA (Poulsen et al. 2012), especially polyunsaturated FA (Keller et al. 1993). Research has shown that the co-expression of SREBP-1 with PPARγ increases the transcriptional activity of PPARγ, even in the absence of ligands for PPARγ (Spiegelman 1998). This interaction of regulators enables efficient lipogenesis in times of a good energy supply. However, if the energy status of cattle becomes poorer, and hence energy is needed, the breakdown of TAG and release of FA, also called lipolysis, occurs (Contreras et al. 2017a). Because of the severe metabolic changes and the onset of lactation and NEB, lipolysis plays an important and overriding role in dairy cows during the periparturient period. Lipolysis not only reduces the size of fat cells by releasing FA, but also leads to a remodeling process that has far-reaching consequences for dairy cattle (Contreras et al. 2017b).

### 2.2.2 Lipolysis

After calving, lipolysis is increased as an adaptation to lactation (Contreras et al. 2017b). The reason for this is that metabolism for the beginning of lactation requires extremely high amounts of energy (De Koster et al. 2016). Furthermore, lipolysis has to increase because the dry matter intake of the cattle is low, resulting in NEB, which must be counteracted (Contreras et al. 2017b).

Lipolysis can be divided into two categories: basal lipolysis, which is influenced by the size of the fat cells and thus the TAG content, and demand lipolysis, which is hormonally regulated when energy is needed (Contreras et al. 2017b). The increase in lipolysis causes a reduction in fat cell size and the release of FA into the bloodstream. These FA are formed by the breakdown of TAG, each of which produces three FA molecules plus one molecule of glycerol (Contreras et al. 2018). The process is initiated by the enzyme adipose triglyceride lipase (ATGL), which acts only on TAG and cleaves them into a diacylglyceride and FFA (Contreras et al. 2018). ATGL, which plays a crucial role in basal lipolysis, is decreased during transition period, indicating that the high demand of energy is counteracted by the action of enzymes involved in demand lipolysis (Koltes 2013; Contreras et al. 2017b). The hormone-sensitive lipase (HSL) then hydrolyzes diacylglycerol to form monoacylglycerols (Contreras et al. 2018). HSL acts more strongly in demand lipolysis than in the basal lipolysis and is furthermore the rate-limiting factor in demand lipolysis; its lipolytic activity is increased during the time after calving (Contreras et al. 2017b). In the last step, FA and glycerol are formed by the action of monoacylglycerol lipase (MGL), which also acts during demand lipolysis (Contreras et al. 2018). The activity and expression of MGL in cattle is unknown, and because of its exclusive hydrolyzation of monoglycerides, the effect of changes on the lipolysis rate are unclear, as HSL is also highly active on monoglycerides (Contreras et al. 2017b). Lipolysis is stimulated when β-adrenergic receptors on the surface of adipocytes are activated by catecholamines such as adrenaline or noradrenaline. These increase the intracellular concentration of cAMP through the conversion of ATP. The increase of cAMP causes the activation of protein kinase A (PKA) which then triggers the lipolysis cascade. Insulin serves as an inhibitory signal of lipolysis (Contreras et al. 2017b). Adipocytes respond highly effectively to insulin, as the binding of insulin to its receptor increases glucose uptake and thus lipogenesis by activating phosphodiesterase 3B (PDE-3B) and hence the degradation of cAMP with subsequently reduced activation of PKA (Contreras et al. 2018). If inhibition of PKA by insulin is missing, a number of molecules, as mentioned above, are phosphorylated and activated, e.g., HSL, and this then increases the lipolytic activity (Contreras et al. 2018). Furthermore, numerous hormonal adjustments in the transition period cause adipocytes to show stronger lipolytic responses, resulting in an increase of NEFA in the circulation. If this increase exceeds a certain level, it leads not only to diseases, like fatty liver as described above, but also to inflammatory responses (Contreras and Sordillo 2011; Contreras et al. 2018).

### 2.2.3 Inflammatory responses during the transition period

Adjustments during the transition period are necessary to prepare cattle for increasing demands with regards to their energy requirements. As mentioned above, NEFA play an important role as an energy source, but excessive lipomobilization can lead to diseases that

are often caused by inflammatory responses (Mavangira and Sordillo 2018). This phenomenon is known from studies on humans, which have shown that elevated concentrations of NEFA increase susceptibility to inflammation-based diseases such as type 2 diabetes or atherosclerosis (Massaro et al. 2008). This effect can also occur in dairy cattle during the transition period and can cause a significant increase in the incidence of diseases such as ketosis, milk fever, mastitis, metritis or displaced abomasum (Contreras and Sordillo 2011; Mavangira and Sordillo 2018). Here, the problem is not only the increase of NEFA, but also the FA composition of the plasma lipid fraction (Contreras and Sordillo 2011). The main FA in the plasma of dairy cows is linoleic acid (Contreras et al. 2010). On the other hand, the FA released during NEB consists mainly of saturated FA, such as palmitic and stearic acid, and the monounsaturated oleic acid (Contreras and Sordillo 2011). Long-chain polyunsaturated FA, such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), which belong to the omega-3 FA, are reduced in the transition period (Contreras and Sordillo 2011). Palmitate and stearate, which are significantly elevated after parturition, are able to increase the activity of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) by activating the inhibitor of the NF-κB kinase (IKKβ), resulting in an increased activity of the transcription factor (Contreras and Sordillo 2011). Direct activation of NF-κB by palmitic or stearic acid activates transcription factors for adhesion molecules, such as the endothelial leukocyte adhesion molecule (ELAM-1). Furthermore, the expression of a variety of cytokines and chemokines is stimulated, which increases the cellular inflammatory response (Contreras and Sordillo 2011). Toll-like receptor 4 (TLR-4)-mediated NF-κB signalling pathway results in an upregulation of proinflammatory genes, causing the elevation of chemokines, cytokines, and reactive oxygen species (ROS) (Sordillo et al. 2009). Furthermore, the activation of TLR-4 also directly affects the insulin transduction pathway by directly inhibiting the phosphorylation of insulin receptor substrate (IRS) by the proinflammatory kinases JNK (c-Jun N-terminal kinase), IKK (IκB kinase), and p38 (p38 mitogen-activated protein kinase) (Kim and Sears 2010) . These factors aggravate the situation of the low availability of insulin in cattle after parturition (Contreras and Sordillo 2011). Omega-3 polyunsaturated FA are able to inhibit the activation of TLR-4 and the inflammatory pathway of NF-κB (Contreras and Sordillo 2011). Furthermore, omega-3 FA, such as linolenic acid or DHA, are able to activate PPARγ, which in turn can inhibit the activation of proinflammatory pathways such as NF-κB (Sordillo et al. 2009). This leads to an anti-inflammatory effect, since cytokines such as IL-1β, IL-6, and TNF-α are not produced (Contreras and Sordillo 2011).

Another critical point during the release of large amounts of FFA is oxidative stress, which occurs when an imbalance arises between oxidants and antioxidants during high metabolic performance (Sies 1997). This leads to far-reaching changes in physiological and metabolic functions (Bernabucci et al. 2005). The most common oxidants are ROS and reactive nitrogen

species (RNS), which can destroy macromolecules consisting of lipids, proteins, and DNA (Mavangira and Sordillo 2018). RNS are derived from nitric oxide synthase reactions (Patel et al. 1999), whereas ROS can originate from the β-oxidation of, for example, palmitate, which of course leads to high rates of oxidants in the case of excessive FA metabolism (Contreras and Sordillo 2011). Increased levels of metabolites from oxidative stress can increase the proinflammatory phenotype of endothelial cells (Sordillo et al. 2009). Furthermore, increased concentrations of RNS and ROS lead to altered leukocyte function (Contreras and Sordillo 2011). Thereby, oxidative stress during the transition period exacerbates inflammatory processes and immune dysfunction (Sordillo and Aitken 2009).

The basic component for the synthesis of lipid mediators (LM) are FA (De Jong et al. 2014) that play a crucial role in pro-inflammatory reactions (Serhan et al. 2008). Lipid mediators include lysophospholipids, phosphoinositides, sphingolipids, eicosanoids, diacylglycerol, ceramides, and phosphatic acids (Contreras and Sordillo 2011). Eicosanoids are one of the main regulators of the acute and chronic inflammatory reactions (Sordillo et al. 2009). These are formed with the help of cyclooxygenase (COX) (Sordillo et al. 2009), whose activity is increased during the transition period (Contreras and Sordillo 2011). Two isoforms of COX are known: COX1 and COX2. COX1 is expressed in small amounts in many tissues and synthesizes small amounts of prostaglandins that are important for physiological body function (Sordillo et al. 2009). COX2, however, is strongly induced by pro-inflammatory stimuli, leading to the synthesis of pro-inflammatory mediators, such as prostaglandin E2 ($PGE_2$), prostaglandin F2α ($PGF_{2\alpha}$), or thromboxane $A_2$ ($TXA_2$) (Sordillo et al. 2009). Furthermore, FA can influence the expression of COX genes through palmitate and stearate which stimulate the expression of COX2 in leukocytes through the activation of TLR-4 (Contreras and Sordillo 2011). This can be downregulated by omega-3 polyunsaturated FA and, hence, high concentrations of saturated FA and lower concentrations of omega-3 polyunsaturated FA increase the expression of COX2 in dairy cattle (Contreras and Sordillo 2011).

Albumin is the most important transport molecule for NEFA (Contreras et al. 2010). It belongs to the negative acute phase proteins and is reduced in the blood during the transition period, as is the case during systemic inflammation (Contreras and Sordillo 2011). An increase in NEFA in the blood, together with the decreased concentration of albumin, leads to an increase in the NEFA to albumin ratio (Contreras and Sordillo 2011). In humans, this increase of ratio is known to cause immune-endothelial dysfunctions such as pre-eclampsia or diabetes type 2 (Contreras et al. 2010). Furthermore, this affects the antioxidant properties of albumin with negative consequences for health (Contreras and Sordillo 2011).

Thus, excessive lipomobilization is of high relevance to the health of cows during the transition period. A better understanding of the mechanisms of lipolysis and the subsequent lipomobilization might probably have positive aspects on health of cattle. In this context,

improved comprehension of the interaction of insulin and the metabolism of adipocytes will play a prominent role.

## 2.3 Magnesium

Magnesium is the eighth most abundant element in the earth's crust (Jahnen-Dechent and Ketteler 2012) and one of the most important elements in the body (Ebel and Gunther 1980); it is an essential ion being the fourth most abundant cation in the body and the second most abundant cation with a variety of functions in the cell (Jahnen-Dechent and Ketteler 2012). The largest reservoir of magnesium, about 60-70%, is present in bones, whereas the remaining 30% lies in the intracellular space (Martens et al. 2018). Only about 1% of magnesium is found extracellularly in serum or red blood cells (Karosanidze 2014). In the body, about 50-70% is ionized, 20-40% is bound to albumin or globulin, and about 10% is bound to anions such as citrate, bicarbonate, or phosphate (Martens et al. 2018). Of these three fractions, the ionized magnesium has the greatest biological activity (Jahnen-Dechent and Ketteler 2012). Magnesium plays an essential role as a cofactor in over 300 enzymatic reactions (Ebel and Gunther 1980), e.g., as an enzyme stabilizer involved in ATP reactions (Jahnen-Dechent and Ketteler 2012). ATP, which is a universal energy carrier, is involved in a variety of reactions, such as glucose, fat, and protein metabolism, the building of nucleic acids, muscle work, and the release of neurotransmitters, which illustrates the essential role of magnesium as a cofactor in vital body functions (Jahnen-Dechent and Ketteler 2012). Moreover, magnesium plays an essential role as a second messenger in the immune system (Martens and Stumpff 2019). The exact regulation of magnesium homeostasis is not yet fully understood, but plasma magnesium is unspecifically influenced by catecholamines, insulin, and parathyroid hormone (PTH) (Martens et al. 2018). Insulin plays a prominent role in cellular magnesium homeostasis as it promotes the uptake of magnesium into the cell, and intracellular magnesium modulates insulin action (Barbagallo et al. 2003). Thus, magnesium homeostasis is tightly regulated at the level of magnesium uptake, magnesium release, and compartmentalization within the cell (Romani 2007). The magnesium concentration is known to be subject to an equilibrium in which its absorption from the intestine is balanced by its excretion from the kidney (De Baaij et al. 2012). Magnesium is absorbed, especially in the small intestine in two ways, namely, a paracellular mechanism driven by the electrochemical gradient and solvent drag, and to a lesser extent but still essential, a transcellular absorption by transient receptor potential channel melastatin member (TRPM) 6 and TRPM7 (Jahnen-Dechent and Ketteler 2012). This is regulated by magnesium dietary uptake and thus availability and by 1,25-dihydroxyvitamin $D_3$ (1,25 $(OH)_2D_3$) (De Baaij et al. 2012). The excretion of magnesium takes place mainly in the kidney. About 90-95% of the daily filtered amount of magnesium is reabsorbed in an

interplay of passive mechanisms and active transporters (De Baaij et al. 2012). The main place of reabsorption is the thick ascending limb of Henle (TAL) in which 60-70% is reabsorbed, whereas the remaining 20-30% is reabsorbed in the proximal tubule (Houillier 2014). The major uptake in TAL is via claudin 16 and 19 (Gunzel and Yu 2013), whereas a smaller residual amount is transported via TRPM6 in the distal convoluted tubule (Houillier 2014). Excretion via the kidneys is subject to circadian rhythm (Jahnen-Dechent and Ketteler 2012) and the regulation of TRPM6 expression by epidermal growth factor (EGF) and estrogen (De Baaij et al. 2012). Magnesium is stored in muscle as a regulator of muscle contraction and in bones as the main store of magnesium in the body (De Baaij et al. 2012). In the event of an acute undersupply, only limited amounts of magnesium can be quickly added to the plasma (Jahnen-Dechent and Ketteler 2012).

In principle, magnesium has been shown to play an essential role in many metabolic processes, so that an undersupply of magnesium has far-reaching consequences for the entire body, especially for glucose metabolism, by influencing insulin pathways.

### 2.3.1 Magnesium homeostasis in cattle

The free ionized magnesium concentration in the plasma of cattle is 0.8-1.2 mmol/L (Martens and Stumpff 2019) and is regulated by intestinal uptake and renal output, as described previously. Because of the binding of magnesium to anions, nucleic acids, or ATP, the intracellular magnesium content is considered to be much higher than the concentration in plasma (Martens and Stumpff 2019). Furthermore, the synthesis of milk plays a major role in magnesium metabolism in cattle. To ensure an adequate supply of magnesium in cattle, absorption from the diet is essential and, in cattle, takes place predominantly in the rumen (Martens et al. 2018). Despite magnesium being also absorbed from the intestine, as described above, this alone would not be sufficient (Fontenot et al. 1989). The absorption of magnesium via the rumen epithelium takes place via potential independent and potential dependent uptake mechanisms (Leonhard-Marek and Martens 1996). At high concentrations of ruminal magnesium, uptake can occur down the electrochemical gradient via pathways that have yet to be identified, but which do not appear to involve paracellular transport. This pathway has high capacity and low affinity and will mediate transport when the ionized concentration of ruminal magnesium is high. In addition to this pathway, absorption of magnesium from the rumen involves a low capacity and high affinity route that is potential dependent. This pathway involves the channels TRPM6 and TRPM7 , which are known as magnesium transporters from the intestine and/or kidney, and whose mRNA and protein have been detected in the rumen epithelium (Martens et al. 2018). The magnesium transporter 1 (MagT1), which mediates $Na^{+}/Mg^{2+}$ exchange, might represent a suitable candidate for basolateral magnesium efflux from the rumen (Schweigel et al. 2008). On the basolateral side, magnesium is then removed

from the cell by the $Na^+/Mg^{2+}$ exchanger SLC41A1 (solute carrier family 41 member 1), the gradient for which is established by the $Na^+/K^+$-ATPase (Martens et al. 2018). However, in many feeding situations, the concentration of ionized magnesium in the rumen drops below the intracellular concentration (0.5 – 1 mmol/L) (Martens et al. 2018). In this situation, uptake of magnesium by the apical membrane has to occur via the high affinity, channel mediated route, and must be driven by the electrochemical gradient (Martens et al. 2018). This is important in so-called grass tetany (hypomagnesemia tetany). Following ingestion of high potassium fodder (for example, young grass in the spring) ruminal potassium concentration rises, leading to a decreased potential difference across the apical membrane. This impedes the resorption of magnesium via the electrochemical gradient via the channel-mediated pathway (Leonhard-Marek and Martens 1996), leading to clinical hypomagnesemia. As in other animals or humans, magnesium is also excreted via the kidneys, as described above. An important factor that reduces the magnesium concentration in the organism is the synthesis of milk, which represents an essential loss in high-performance cattle (Martens et al. 2018). The concentration of magnesium in milk (on average 108 mg/L (Cerbulis and Farrell 1976)) is higher than that in serum, and the concentration of magnesium in colostrum is again much higher (on average 733 mg/kg (Kehoe et al. 2007)), which illustrates the importance of magnesium, especially during parturition.

Due to the previously mentioned mechanisms, it can be hypothesized that cattle with an undersupply of magnesium quickly reach their limits with far-reaching consequences for many metabolic problems and diseases, which can worsen the already difficult situation of the transition period.

### 2.3.2 Interaction of magnesium and insulin

The importance of magnesium in many metabolic processes has been demonstrated in the previous sections. For some years now, the interaction of magnesium and insulin has been a widely researched field with many studies and findings (Paolisso et al. 1990; Alzaid et al. 1995; Takaya et al. 2004; De Lourdes Lima et al. 2009; Mastrototaro et al. 2015). Because of the ever-increasing number of cases of type 2 diabetes in humans and the associated diseases, such as cardiovascular disorders, this topic has become the focus of extensive research. Insulin resistance and lipid metabolism disorders are also known to occur in type 2 diabetes mellitus in humans and have been increasingly correlated with magnesium deficiency during the last few years. A large multicenter study by Kao et al. in 1999 described for the first time an inverse relationship between serum magnesium levels and the incidence of type 2 diabetes mellitus (Kao et al. 1999). Further studies showed that both total and ionized magnesium levels in serum were decreased in 25-39% of diabetic patients (De Valk 1999). When insulin binds to its receptor, a series of phosphorylation reactions occurs, for example, with regard to various

kinases or the insulin receptor substrates (IRS 1-6). Here, magnesium acts together with ATP as a kinase substrate and as a second messenger at the insulin receptor tyrosine kinase (IRTK) (Gunther 2010). Thus, the relationship between magnesium availability and insulin sensitivity is reciprocal: an evolved magnesium deficiency inhibits the tyrosine kinase activity of the insulin receptor and signal transduction at the post-receptor level and thus can exacerbate existing insulin resistance (Barbagallo et al. 2003). On the other hand, insulin regulates the cellular magnesium balance and leads to increased intracellular magnesium content (compared with the extracellular space) in healthy individuals (Paolisso et al. 1990). This interaction illustrates the complexity of this system, because insulin increases magnesium uptake into the cell, whereas intracellular magnesium deficiency forces insulin resistance (Volpe 2008). A direct correlation also exists between magnesium availability and fat metabolism. Adipocytes can have an endocrine effect by releasing effectors such as interleukins or TNF-α or even catecholamines, especially in the case of magnesium deficiency, which in turn increases insulin resistance (Gunther 2010). Another worsening effect is seen in lipolysis, which results in the release of NEFA. Kurstjens et al. have been able to show that increased triglyceride and NEFA levels directly reduce the amount of free magnesium in the blood, since magnesium is bound to lipids and is therefore no longer available for the cells (Kurstjens et al. 2019). Possibly, this could cause a shift of magnesium from intracellular pools into the blood stream to maintain the blood concentration of magnesium, which in turn decreases insulin sensitivity and further depletes the cell of magnesium. All in all, the description so far shows that the interaction of magnesium and insulin can lead to a vicious circle of insulin resistance in cases of metabolic imbalances. Therefore, in humans, magnesium supplementation in excess of requirements is recommended to improve glucose (Veronese et al. 2016) and lipid metabolism in diabetes (Verma and Garg 2017).

Although the condition of diabetes type 2 in humans is intriguingly similar to the transition period in cattle, no knowledge is available on this subject in cattle. A better understanding of all these factors would represent a breakthrough in the treatment of the typical diseases of high-yielding cattle. This includes an understanding of the consequences of bovine metabolic disorders on insulin action and lipid metabolism and thus its effect on adipocytes. Because of the outstanding importance of magnesium in all these processes, improvements in the insulin signaling pathway, especially in adipocytes, via enhancement of the magnesium status might represent a promising solution to the problem of the transition period of cows.

## Chapter 3: Aims and objectives of this thesis

In the last decades, research into bovine metabolic diseases during the transition period has gained increasing interest; however, many important questions remained open. The interplay of lipolysis, the accompanying massive lipomobilization, and insulin resistance are now known to play significant roles. The effect of magnesium in these processes is well studied in humans suffering from diabetes type 2 but not in cattle.

The central hypothesis of this project is that the excessive fat mobilization in high-yielding cows post-partum is to a significant extent the consequence of inefficient insulin signaling with far-reaching consequences. Consequently, the interaction of insulin signaling with magnesium homeostasis has been investigated here in a bovine adipocyte model by using various molecular and functional techniques. Of central importance was the question as to whether insufficient insulin signaling can be at least partially compensated by higher amounts of magnesium. The findings should open new therapeutic approaches in the prophylaxis and treatment of ketosis and fatty liver syndrome in high-yielding cows.

The first series of *in vitro* experiments investigated the influence of various medium additives on the cultivation and differentiation of bovine adipocytes and sought to establish a successful cultivation protocol. The following special subjects were further addressed: *(i)* the influence of bovine serum lipids (BSL) and fetal bovine serum (FBS) on the growth and differentiation of cultured bovine adipocytes and *(ii)* the change in the expression pattern of surface markers, such as stem cell markers or markers of differentiated adipocytes, and the dependence of these changes on the addition of BSL or FBS as an indication of the effect of the medium additives on the development of cultured adipocytes.

The second series of experiments focused on the optimization of adipogenic transdifferentiation of bovine mesenchymal stem cells. Here, the aim was to investigate the influence of several medium additives on the induction and differentiation of mesenchymal stem cells into functional adipocytes. Specific topics were: *(i)* the influence of ascorbic acid, bovine serum lipids (BSL), fetal bovine serum (FBS), glucose, and acetic acid on transdifferentiation into adipocytes, *(ii)* the influence of these factors on the accumulation of intracellular lipids, the expression of stem cell and adipocyte markers, and their relationships and correlated changes during transdifferentiation, and *(iii)* the influence of various coatings of the cell culture plates to prevent detachment after differentiation by the buildup of lower density fat vacuoles and thus to avoid the floating of cells.

After the establishment of a cultivation protocol of adipocytes with evidence of transdifferentiation, the influence of various magnesium concentrations and insulin concentrations was investigated with respect to the central metabolic functions of bovine adipocytes. The question was whether an adequate or increased magnesium concentration

could compensate for a possible insufficient insulin signal. Various insulin and magnesium concentrations were tested on cultivated bovine adipocytes in order to investigate *(i)* the accumulation of non-polar lipids in differentiated bovine adipocytes, *(ii)* the glucose uptake via the glucose transporter GLUT4, *(iii)* the activation of glycerol-3-phosphate dehydrogenase (GPDH) representing the link between carbohydrate and lipid metabolism, and *(iv)* the expression of magnesium-responsive genes in order to investigate the possible up- or down-regulation of those genes relevant to magnesium homeostasis but dependent on the insulin and/or magnesium supply.

# Chapter 4: Influence of bovine serum lipids and fetal bovine serum on the expression of cell surface markers in cultured bovine preadipocytes

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# Optimizing adipogenic transdifferentiation of bovine mesenchymal stem cells: a prominent role of ascorbic acid in *FABP4* induction

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RESEARCH PAPER

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# Optimizing adipogenic transdifferentiation of bovine mesenchymal stem cells: a prominent role of ascorbic acid in *FABP4* induction

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**ABSTRACT**

Adipocyte differentiation of bovine adipose-derived stem cells (ASC) was induced by foetal bovine serum (FBS), biotin, pantothenic acid, insulin, rosiglitazone, dexamethasone and 3-isobutyl-1-methylxanthine, followed by incubation in different media to test the influence of ascorbic acid (AsA), bovine serum lipids (BSL), FBS, glucose and acetic acid on transdifferentiation into functional adipocytes. Moreover, different culture plate coatings (collagen-A, gelatin-A or poly-L-lysine) were tested. The differentiated ASC were subjected to Nile red staining, DAPI staining, immunocytochemistry and quantitative reverse transcription PCR (for *NT5E, THY1, ENG, PDGFRα, FABP4, PPARγ, LPL, FAS, GLUT4*). Nile red quantification showed a significant increase in the development of lipid droplets in treatments with AsA and BSL without FBS. The presence of BSL induced a prominent increase in *FABP4* mRNA abundance and in FABP4 immunofluorescence signals in coincubation with AsA. The abundance of *NT5E, ENG* and *THY1* mRNA decreased or tended to decrease in the absence of FBS, and ENG was additionally suppressed by AsA. DAPI fluorescence was higher in cells cultured in poly-L-lysine or gelatin-A coated wells. In additional experiments, the multi-lineage differentiation potential to osteoblasts was verified in medium containing ß-glycerophosphate, dexamethasone and 1,25-dihydroxyvitamin $D_3$ using alizarin red staining. In conclusion, bovine ASC are capable of multi-lineage differentiation. Poly-L-lysine or gelatin-A coating, the absence of FBS, and the presence of BSL and AsA favour optimal transdifferentiation into adipocytes. AsA supports transdifferentiation via a unique role in *FABP4* induction, but this is not linearly related to the primarily BSL-driven lipid accumulation.

**Abbreviations**: AcA: acetic acid; AsA: ascorbic acid; ASC: adipose-derived stem cells; BSL: bovine serum lipids; DAPI: 4′,6-diamidino-2-phenylindole; DLK: delta like non-canonical notch ligand; DMEM: Dulbecco's modified Eagle's medium; DPBS: Dulbecco's phosphate-buffered saline; ENG: endoglin; FABP: fatty acid binding protein; FAS: fatty acid synthase; GLUT4: glucose transporter type 4; IBMX: 3-isobutyl-1-methylxanthine; LPL: lipoprotein lipase; MSC: mesenchymal stem cells; α-MEM: α minimum essential medium; NT5E: ecto-5′-nucleotidase; PDGFRα: platelet derived growth factor receptor α; PPARγ: peroxisome proliferator activated receptor γ; RPS19: ribosomal protein S19; SEM: standard error of the mean; THY1: Thy-1 cell surface antigen; TRT: treatment; TRT-Con: treatment negative control; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

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## Introduction

Adult stem cells are undifferentiated cells that can continue their self-renewal for extended periods of time. Upon specific stimuli, they can undergo self-transformation into specialized cell types [1]. Adipose-derived stem cells (ASC) are present in many fat depots of the body and appear as fibroblast-like cells upon culture. The International Fat Applied Technology Society has stated that the term ASC should be used for committed adipogenic progenitors (pre-adipocytes). Many tissues of human and animal origin have been employed as sources of ASC, e.g. subcutaneous [2], intraperitoneal [3], muscle [4], visceral [5] and bone marrow tissues [6]. Thus, a wide array of adult adipose tissues can serve as a pool for adipocyte progenitors. Pre-adipocytes are capable of multi-lineage differentiation into myocytes, osteoblasts, chondrocytes and adipocytes [7]. This multipotency of ASC has made them focus of much research [8]. The transformation of ASC into fully functional adipocytes is dependent upon the expression

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of special cellular markers. Cell surface markers such as ecto-5′-nucleotidase (NT5E, CD73), Thy-1 cell surface antigen (THY1, CD90), platelet derived growth factor receptor *α* (PDGFR*α*) and endoglin (ENG, CD105) allow the discrimination of ASC from other differentiated cell types [9,10]. The major characteristics of ASC are their adherence to plastic, their production of specific cell surface markers, and their differentiation capability as described by the International Society of Cell Therapy [11]. ENG is an adhesion molecule that plays an important role in transforming growth factor β signalling, which may trigger pre-adipocyte differentiation into chondrocytes [9]. NT5E is an ecto-5′-nucleotidase surface enzyme that has a role in cell-to-cell interactions [10] and that is also involved in mesenchymal stem cell immunity [12]. THY1 is a less well defined ASC marker and is involved in cell-to-cell interactions [13]. PDGFR*α*, a cell surface tyrosine kinase receptor, is expressed on cells that fulfill the definition of MSC and induces many effects, like cell proliferation or transformation [14].

To date, diverse types of hormonal and chemical cocktails have been suggested for the induction and further transformation of ASC into mature adipocytes with variable success, most of them containing foetal bovine serum (FBS), biotin, pantothenic acid, insulin, rosiglitazone, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) during the induction phase [15,16]. For bovine ASC differentiation, the addition of insulin and ascorbic acid (AsA) has been used at various stages of adipocyte culture [17]. Similarly, bovine serum lipids (BSL), a triglyceride-rich cell culture supplement from bovine blood, has shown a potent effect on the differentiation of intramuscular and subcutaneous pre-adipocytes into adipocytes [18]. In the experiments described herein, we intended to specifically titrate the supplement concentrations of induction and differentiation media to the needs of bovine subcutaneous ASC. Such a model system would be very helpful for studying the regulation of lipid metabolism in dairy cows. The latter may be different to humans and monogastric animals; because dairy cows have lower blood glucose levels and drive adipogenesis in adipose tissue primarily from blood acetic acid (AcA) [19]. Therefore, the bovine ASC might be a valuable model system for comparative basic research. As we have previously shown that the adipogenesis of bovine ASC is promoted by the presence of BSL and the absence of FBS [20], we wondered whether AsA may also affect adipogenic differentiation at various levels of BSL and FBS. Furthermore, we hypothesized that, for bovine ASC, AcA is an important nutrient promoting adipogenesis and that AcA effects are dependent upon glucose availability. In addition to testing these hypotheses, the methodological aims of our study were to optimize the induction and differentiation media for bovine ASC, to determine the effects of various plastic coating materials on cellular adhesion and to verify the multi-lineage potential of the used ASC by demonstrating their transdifferentiation potential towards osteoblasts.

## Materials and methods

### Materials

FBS, BSL (Ex-Cyte), trypsin-EDTA, penicillin-streptomycin, AcA, Dulbecco's phosphate-buffered saline (DPBS), collagen-A, and the cell culture media DMEM and complete α minimum essential medium (α-MEM) were obtained from Merck Millipore (Darmstadt, Germany). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), amphotericin B, AsA, biotin, pantothenic acid, bovine insulin, rosiglitazone, dexamethasone, IBMX, Nile red, Triton$^{TM}$ X-100, gelatin-A, poly-L-lysine, ß-glycerophosphate, 1,25-dihydroxyvitamin $D_3$, alizarin red (sodium sulphonate salt) and D-glucose were purchased from Sigma Aldrich (Taufkirchen, Germany). Mount Fluor was supplied by BioCyc GmbH & Co. (Luckenwalde, Germany) and the fixative solution Roti-Histofix 4% was from Carl Roth (Karlsruhe, Germany). The antibodies against NT5E, THY1, delta like non-canonical notch ligand 1 (DLK1), fatty acid binding protein 4 (FABP4), glucose transporter type 4 (GLUT4) and fatty acid synthase (FAS) were from AbCam (Cambridge, UK), whereas the antibody against platelet derived growth factor receptor α (PDGFRα) was obtained from Biorbyt Ltd. (Cambridge, UK). The antibody against ENG was purchased from Thermo Scientific (Massachusetts, USA) and 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Roche (Grenzach-Wyhlen, Germany). Other consumables are detailed at other places. Cell culture plates and other plastic ware were from Techno Plastic Products, Trasadingen, Switzerland or resources stated elsewhere.

### Adipose tissue assortment

Tissue harvest was in accordance with the German legislation on animal welfare; however, no Animal Use and Care approval was required because no animals were specifically reared or killed for the present experiments. Samples of bovine subcutaneous adipose tissue were aseptically collected from the neck region (2$^{nd}$ to 3$^{rd}$ vertebrae) of exsanguinated Holstein calves (< 11 months old) from

a local abattoir. After collection, adipose tissue was washed with sterile calcium- and magnesium-containing DPBS and transferred to ice-cold DPBS with the antibiotics penicillin-streptomycin (400 U/mL and 400 µg/mL, respectively) and nystatin (240 U/mL). Incubation in this antibiotic-supplemented DPBS continued during the transport of the tissue to the Institute of Veterinary Physiology, Freie Universität Berlin (approximately 1 h).

### Explant culture

As previously reported [3,20], the subcutaneous adipose tissues were washed with fresh DPBS and cut into 2–3 $mm^3$ blocks without visible blood vessels. The blocks were placed at equal distances of ~5 mm in 6-well cell culture plates with 0.5 mL/well DMEM with 4 mM L-glutamine, 20% FBS, penicillin-streptomycin (100 U/mL and 100 µg/mL), and amphotericin B (2.5 µg/mL), referred to as the base medium hereafter. After incubation in a humidified atmosphere at 5% $CO_2$ and 37°C, a quantity of 1.5 mL pre-warmed base medium was added per well after the explants had attached to the bottom of each well (~48 h). The medium was replaced every 48 h, taking care that explant tissues were not washed away or floating on the medium. On the expansion of fibroblast-like cells, the explants were removed on the $7^{th}$ day, and cells were left to grow and become confluent. This cell culture is referred to as passage number zero (P0).

### Cell passaging

For the separation of cells from confluent ASC monolayers, the cells were washed with DPBS (without Ca and Mg) and incubated with 0.5 mL trypsin-EDTA (0.25% and 0.02%, w/v respectively) in PBS (without Ca and Mg) in a 5% $CO_2$ incubator at 37°C for 10 min. After the detachment of the cells, 5 mL fresh DMEM medium with 10% FBS was added to stop the enzymatic trypsin action. The cells were transferred into T25 culture flasks supplemented with pre-adipocyte cell culture medium (DMEM with 10% FBS, penicillin-streptomycin (100 U/mL and 100 µg/mL), amphotericin B (2.5 µg/mL), and 15 mM HEPES). Cells were maintained in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. When cells were 85 – 90% confluent, ASC were trypsinized again, and this passage cycle was repeated in new T75 flasks until P5. Subsequently, the cells were counted manually in a Neubauer chamber and viability was assessed by the trypan blue exclusion test (> 95%). For the experiments, ASC ($1.5 \times 10^4$ cells/well) were transferred into 24-well cell culture plates with or without coverslips, the former being dedicated for immunohistochemistry staining, the latter for the measurement of lipid content and quantitative reverse transcription PCR. All further experiments were carried out with ASC at P6.

### Validation of ASC identity and multi-lineage potential

The ASC were kept in 24-well cell culture plates with coverslips and pre-adipocyte medium as described above until the cells had reached confluence with two additional days under conditions of growth arrest. Afterwards, the cells were immersed in adipocyte induction medium for 2 days. Adipocyte induction medium consisted of (if not stated otherwise) DMEM with 4 mM L-glutamine, supplemented with HEPES (15 mM), biotin (10 µM), bovine insulin (3 µg/mL), dexamethasone (0.3 µM), IBMX (0.1 mM), rosiglitazone (10 µM), penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), and amphotericin B (2.5 µg/mL). Thereafter, the medium was changed to differentiation medium, which consisted of DMEM with 4 mM L-glutamine, penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), amphotericin B (2.5 µg/mL), HEPES (15 mM), biotin (10 µM), and bovine insulin (3 µg/mL). Differentiation medium was applied for 7 or 14 days with culture medium exchange after every 48 h. The cells were washed with DPBS, fixed in 4% Roti-Histofix for 30 min, and assessed by phase contrast microscopy in order to evaluate the accumulation of triglycerides and phenotypic changes in cell morphology. The ASC were further examined using fluorescent immunocytochemistry staining against NT5E, THY1, ENG, DLK1, PDGFRα, GLUT4, FAS and FABP4 markers. The staining procedure and the use of specific antibodies are detailed in the next subsections.

To verify the multi-lineage potential of bovine ASCs, $1.35 \times 10^5$ cells/well were plated in 6-well culture plates and treated with complete α-MEM. After reaching confluency, the medium was switched to osteogenic differentiation medium (complete α-MEM supplemented with 10 mM ß-glycerophosphate, 0.1 mM dexamethasone and 5 nM of 1,25-dihydroxyvitamin $D_3$) for 21 days. Ascorbic acid (300 nM) was also present in the medium. The control cells were kept in complete α-MEM without osteogenic stimulants. After 21 days, culture medium was removed, cells were washed twice with DPBS, fixed with 4% Roti-Histofix for 30 min. The working solution of alizarin red stain (2 g alizarin red in 100 mL distilled water, pH 4.3) was added for 45 min at room temperature and cells were washed with DPBS. After staining, alizarin red was visualized using an inverted light microscope (Motic AE2000).

### *Staining and evaluation of non-polar lipids*

The lipid contents of the cells were assessed by Nile red staining. Briefly, the medium was removed from the culture wells, and the cells were washed twice with cold DPBS for 5 min. Thereafter, the cells were fixed in Roti-Histofix 4% solution for 30 min at room temperature and washed twice in DPBS for 5 min each. A final concentration of Nile red of 5 µg/mL (in DPBS) was used, in the dark for 5 min, to stain cellular lipids. Subsequently, the cells were treated with 0.5% Triton™ X-100 solution in the dark for 5 min and thereafter stained with 0.2 µg/mL of DAPI in the dark for 5 min. The fluorescence indices for Nile red and DAPI were measured by an EnSpire Multimode Plate Reader (PerkinElmer, Massachusetts, USA). For Nile red, two sets of wavelengths were used as suggested by Greenspan et al. [21]. These were (excitation/emission) 515/590 nm to visualize total lipids and 475/570 nm to selectively visualize non-polar lipids. For DAPI the excitation/emission was 358/461 nm. The fluorescence signal for Nile red (lipid index) was divided by DAPI fluorescence (nuclei index) to obtain a lipid/nuclei index in which the Nile red fluorescence was corrected for variation in cell density. The same staining protocols were applied to cells grown on 24-well plates for microscopic visualization of Nile red and DAPI fluorescence by using a Leica DMI 6000B epi-fluorescent microscope with a 20× objective (Leica microsystems, Wetzlar, Germany).

### *Immunofluorescence cytochemistry*

ASC were grown on round sterilized glass cover slips (6 mm). Using dual-colour immunocytochemistry, the cells were characterized for the presence of the ASC cell surface markers (NT5E, THY1, ENG, DLK1, PDGFRα) and markers of differentiated adipocytes (FABP4, GLUT4 and FAS). Briefly, the cells were washed with cold DPBS (with Ca and Mg), fixed in Roti-Histofix 4% solution for 30 min, and washed twice in DPBS for 5 min. Next, the cell membranes were permeabilized by incubation of cells with 0.3% Triton™ X-100 in DPBS for 20 min, followed by an incubation in 5% goat serum for 25–30 min. Thereafter, the cells were incubated with primary and secondary antibodies as detailed in Table 1. Nuclei were counterstained with DAPI (0.2 µg/mL) at room temperature for 5 min in the dark. The cells were washed twice with DPBS, mounted on clean glass slides with Mount Fluor (BioCyc, Potsdam, Germany), and stored at 4°C. Negative controls were incubated with DPBS instead of the primary antibodies while the rest of the protocol remained unchanged. Fluorescence signals were evaluated on a Leica DMI 6000B epi-fluorescent microscope with a 63× objective.

### *RNA isolation and quantitative real-time polymerase chain reaction*

For RNA isolation, the cultured ASC before induction and after the development of adipocytes (14 days) were washed two times with DPBS and trypsinized. Cells were resuspended in DPBS, centrifuged at 300 × *g* at 4°C for 5 min and the cell pellet was stored at −80°C in RNAlater® (Invitrogen, California, USA). The NucleoSpin® RNA kit (Machery-Nagel GmbH & Co., Düren, Germany) was used to extract total RNA according to the manufacturer's instructions. The quantity of RNA was assessed at 260 nm by using a Nano-Photometer (Implen, Munich, Germany).

**Table 1.** Characteristics and details of antibodies used, together with their antigens.

| Antigens | Primary antibodies | Incubation protocol (primary antibody) | Secondary antibodies[a] | Visualization (excitation/ emission) |
|---|---|---|---|---|
| NT5E | Rabbit polyclonal, (ab137595), 1:100 | At room temperature for 1 h and then left overnight at 4°C in a humidified chamber | Goat anti rabbit IgG, labeled with Alexa Fluor 488 | 489 nm/508 nm |
| THY1 | Rabbit monoclonal, (ab92574), 1:50 | | | |
| DLK1 | Rabbit polyclonal, (ab21682), 1:200 | | | |
| FABP4 | Rabbit polyclonal, (ab85875), 1:100 | | | |
| ENG | Mouse monoclonal, (MA5-11854), 1:50 | At room temperature for 1 h and then left overnight at 4°C in a humidified chamber | Goat anti mouse IgG, labeled with Alexa Fluor 594 | 596 nm/620 nm |
| PDGFR-α | Rabbit polyclonal (orb6660), 1:50 | At 37°C temperature for 1 h and then left overnight at 4°C in a humidified chamber | Goat anti rabbit IgG, labeled with Alexa Fluor 488 | 489 nm/508 nm |
| GLUT4 | Rabbit polyclonal (ab33780), 1:30 | | | |
| FAS | Rabbit polyclonal (ab22759), 1:50 | | | |

[a]Incubation with secondary antibodies was at room temperature for 45 min.

Table 2. Primers used to amplify specific genes of the bovine subcutaneous ASC and differentiated adipocytes.

| Gene | Sense (5′-3′) | Anti-sense (3′-5′) | Amplicon size (bp) | RefSeq identifier |
|---|---|---|---|---|
| *NT5E* | TTTGGAGGCACCTTTGACC | AGAGGCTCATAACTGGGCAC | 212 | NM_174129.3 |
| *THY1* | CAACTTCACCACCAAGGATG | TCTGGATCAGCAGGCTTATG | 140 | NM_001034765.1 |
| *ENG* | CCTCAGCGTGAACAAATCC | CGTGAAAGACCAGTTTGGAG | 89 | NM_001076397.1 |
| *FABP4* | TGGGATGGAAAATCAACCAC | TGGCTTATGCTCTCTCATAAAC | 112 | NM_174314.2 |
| *RPS19* | GGAAAAGGACCAAGATGGGG | CGAACGAGGCAATTTATTAACC | 136 | NM_001037467.2 |
| *YWHAZ* | AGAGAGAAAATAGAGACCGAGC | AGCCAAGTAGCGGTAGTAG | 144 | NM_174814.2 |
| *GLUT4* | AGCCATGAGCTATGTCTCC | AAGATGAAGAAGCCAAGCAG | 255 | NM_174604.1 |
| *PPARγ* | TAAAGAGCCTGCGAAAGCC | GCTTCACGTTCAGCAAACC | 156 | NM_181024.2 |
| *LPL* | AGAGTAAAGGCAGGAGAGAC | CAGCCAGACTTTCTATTCAGG | 134 | NM_001075120.1 |
| *PDGFRα* | CATCTTTGACAACCTGTACACC | TAGAGTCCACCATCATGCC | 113 | NM_001192345.3 |
| *GAPDH* | AAGAAGGTGGTGAAGCAGG | GCATCGAAGGTAGAAGAGTGAG | 116 | NM_001034034.2 |

Total RNA (100 ng/µL) was reverse transcribed by using an iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). Quantitative reverse transcription PCR was carried out in an Viia7 real time PCR cycler (Thermo Scientific, Massachusetts, USA) with SYBR Green master mix (Bio-Rad, Munich, Germany) and the gene-specific, intron spanning primers for *NT5E, THY1, ENG, PDGFRα, PPARγ, LPL, GLUT4* and *FABP4* presented in Table 2. Amplification of cDNA was carried out in a final volume of 10 µL containing 5 µL mastermix, 1 µL primer sense, 1 µL primer antisense, and 3 µL cDNA. The temperature protocol consisted of an initial denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 30 sec. PCR was followed by a melting curve analysis to validate specificity. The $C_t$ values of the target genes were normalized to ribosomal protein S19 (*RPS19*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). All reactions were performed in triplicate and relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method [22]. Negative controls without cDNA template were included in all reactions.

### Experimental setups to optimize adipogenic differentiation

In total, three experimental setups were conducted to identify the best protocol for the rapid conversion of bovine ASC to mature adipocytes.

#### Experiment 1: optimization of the induction medium for bovine ASC

The first trial was carried out to investigate the minimal working concentration of adipogenic supplements in the induction medium. The basal induction medium was DMEM (with 4 mM L-glutamine) supplemented with FBS (10%), pantothenic acid (17 µM), biotin (33 µM), insulin (10 µg/mL), dexamethasone (1 µM), rosiglitazone (20 µM) and IBMX (250 µM) as stated by Riedel et al. [23]. This medium was referred to as the 100% medium. In parallel incubations, the concentrations of pantothenic acid, biotin, bovine insulin, dexamethasone, IBMX and rosiglitazone were reduced to 30% and 10% of the 100% medium, and the transformation of bovine pre-adipocytes into adipocytes was assessed by using Nile red staining of non-polar lipids.

#### Experiment 2: in vitro induction and differentiation potential of bovine ASC

To determine the adipogenic effects of FBS, BSL and AsA on ASC, a second experiment was conducted on subcutaneous ASC isolated from three different animals. In 24-well cell culture plates, confluent ASC that had undergone growth arrest were treated with 30% induction medium, i.e. DMEM (with 4 mM L-glutamine) supplemented with FBS (10%), HEPES (15 mM), biotin (10 µM), bovine insulin (3 µg/L), dexamethasone (0.3 µM), IBMX (0.1 mM), rosiglitazone (10 µM), penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), and amphotericin B (2.5 µg/mL). After 2 days of ASC induction, the cells were kept in seven different types of adipocyte differentiation media in which FBS, BSL or AsA were either present or absent for either 7 or 14 days. The code used to identify the treatments (TRT) is presented in Table 3. Adipogenic differentiation was assessed by using Nile red staining, immunocytochemistry and quantitative reverse transcription PCR.

#### Experiment 3: effect of AcA, glucose and various coatings on adipogenesis

ASC were cultured in 24-well cell culture plates that were either left non-coated or coated with collagen-A, gelatin-A or poly-L-lysine. After reaching confluence

Table 3. Experimental concentrations of ascorbic acid (AsA), foetal bovine serum (FBS) and bovine serum lipids (BSL) in the differentiation media used in Experiment 2.

| Treatments | AsA (µg/mL) | FBS (%) | BSL (µL/mL) |
|---|---|---|---|
| TRT-Con | 0 | 0 | 0 |
| TRT-1 | 0 | 10 | 0 |
| TRT-2 | 0 | 10 | 10 |
| TRT-3 | 0 | 0 | 10 |
| TRT-4 | 40 | 10 | 0 |
| TRT-5 | 40 | 10 | 10 |
| TRT-6 | 40 | 0 | 10 |

with two additional days under growth arrest, the ASC were kept in induction medium for 2 d. Two experimental runs with 4 replicates were conducted with various differentiation media based on the following components: DMEM without glucose but with L-glutamine (4 mM), penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively), amphotericin B (2.5 μg/mL), HEPES (15 mM), biotin (10 μM), insulin (3 μg/mL), AsA (113 μM), and BSL (5 μL/mL). The treatment media were then supplemented with 10 or 25 mM glucose together with 0, 10 or 20 mM AcA. Adipogenic effects were determined by using Nile red staining after 7 and 14 d in differentiation medium.

### *Statistics*

All data sets presented in the manuscript were statistically analysed and graphs were plotted by using SigmaPlot 11.0 software (Systat Software Inc., San Jose, CA, USA). All experiments were conducted in triplicate with three to five different animals. Each observation was derived from duplicate (Experiments 2, 3) or triplicate (Experiment 1) measurements that were arithmetically pooled from 2 or 3 wells of a 24-well culture plate. Each animal was considered as an experimental unit. In Experiments 1 and 2, the effects of different supplements were investigated by two-way ANOVA with fixed effects of treatment and days of treatment, except for the quantitative reverse transcription PCR data, which was evaluated by one-way ANOVA. In Experiment 3, a three-way ANOVA was applied, and least square means were computed for the fixed effects of supplement treatments, coating materials, and days of treatment with their two- and three-way interactions. If ANOVA indicated a significant effect, differences among groups were identified by the Holm-Sidak or Dunn's post-hoc test. All the data are presented as means ± standard errors of the mean (SEM); $P < 0.05$ was considered statistically significant.

## Results

### *Verification of ASC identity*

All adipose tissue explants were kept in 6-well tissue culture plates with a limited volume of culture medium to maintain them in permanent contact with the culture surface. Within 3–5 d, fibroblast-like cells started emerging from the tissue explants (Figure 1(a)). The cells were identified by their characteristic spindle shape. Upon transfer of the cells to induction medium, the ASC rapidly started to differentiate and to accumulate fat as shown in Figure 1(b).

For further identification of ASC, immunocytochemistry for well-defined ASC markers was carried out and revealed the presence of NT5E, THY1, and ENG (Figure 1(c,d)). NT5E was located around the nucleus and was most probably located in the Golgi apparatus, whereas the presence of THY1 and ENG was diffuse throughout the cell and in the cell membrane.

In addition, to demonstrate the multi-lineage potential of ASC, we differentiated the ASC to osteoblasts. Upon cultivation of the ASC in the appropriate differentiation medium, calcification was visualized using staining with alizarin red, a commonly used dye to stain calcium deposits. As shown in Figure 1, control cells incubated in the absence of osteogenic stimulants did not show any staining (Figure 1(e)) while cells kept in osteogenic differentiation medium accumulated minerals as demonstrated by dense staining that appeared red under the inverted light microscope (Figure 1(f)).

### *Experiment 1: optimization of induction medium for bovine ASC*

The induction of pre-adipocytes differentiation critically depends upon the parallel stimulation of insulin, glucocorticoid and peroxisome proliferator-activated receptor (PPAR)γ, and is greatly enhanced by cyclic adenosine monophosphate (cAMP) [24]. Thus insulin, dexamethasone, rosiglitazone, and cAMP-stimulating agents such as IBMX, are usually supplied in massively supraphysiological concentrations in induction media for adipogenic differentiation. To elucidate whether these extremely high concentrations are optimal for bovine ASC, we proportionally reduced the concentrations of supplements starting from '100% medium' containing 10 μg/mL insulin, 1 μM dexamethasone, 20 μM rosiglitazone, 250 μM IBMX and 33 μM biotin to medium containing only 30% or 10% of each of these supplements. At all three concentrations, a rapid accumulation of cellular lipid occurred by day 5 ($P < 0.001$ to zero; Figure 2), indicating that only a few days in induction medium were sufficient for adipogenic induction in bovine ASC. Lipid accumulation between day 5 and day 35 was affected by the factor day of treatment ($P < 0.001$), with lipid accumulation on day 14, day 28, and day 35 being higher than that on day 5 ($P < 0.05$; Figure 2). The effect of treatment was not significant ($P > 0.05$); however, the '10% medium' appeared to trigger lower lipid accumulation

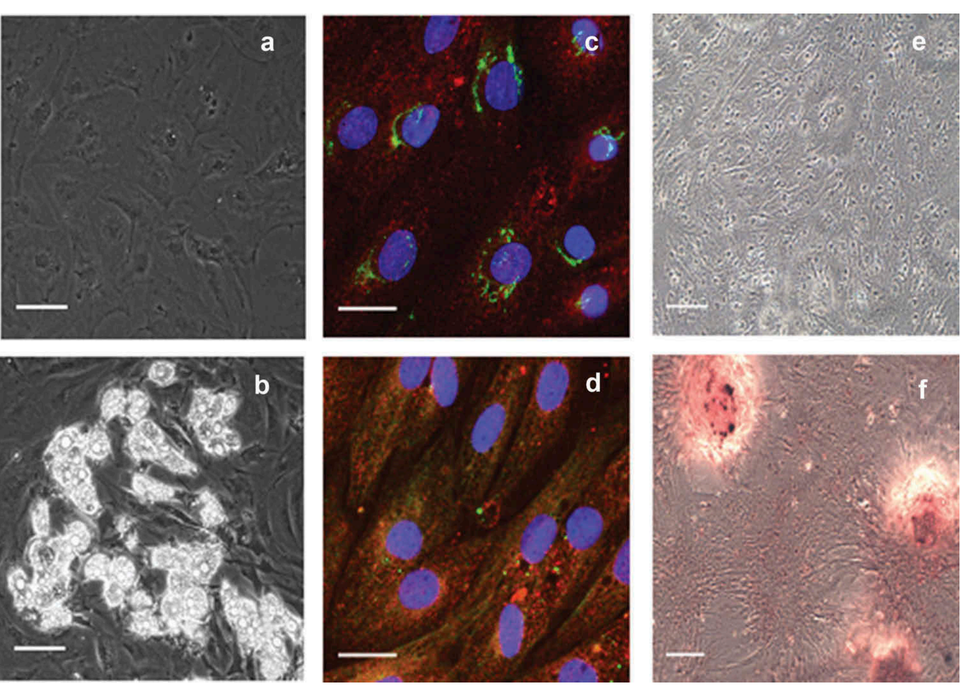


**Figure 1.** Verification of ASC identity. Representative phase contrast microscopic images of (a) pre-adipocytes before induction and (b) developed adipocytes after induction and 14 days in differentiation medium. Lipid droplets are amply present in differentiated adipocytes of graph b. The immunocytochemistry of undifferentiated pre-adipocytes identifies (c) the presence of NT5E (green) and ENG (red), as well as (d) recognition of THY1 (green) and ENG (red). For comparison, inverted light microscopic images using alizarin red staining are shown after 21 days (c) in control medium or (d) in osteogenic differentiation medium. The scale bar is representative of 100 µm in panels a and b (using a 20× objective), 25 µm in panels c and d (using a 63× objective), and 100 µm in panels e and f (using a 10× objective).

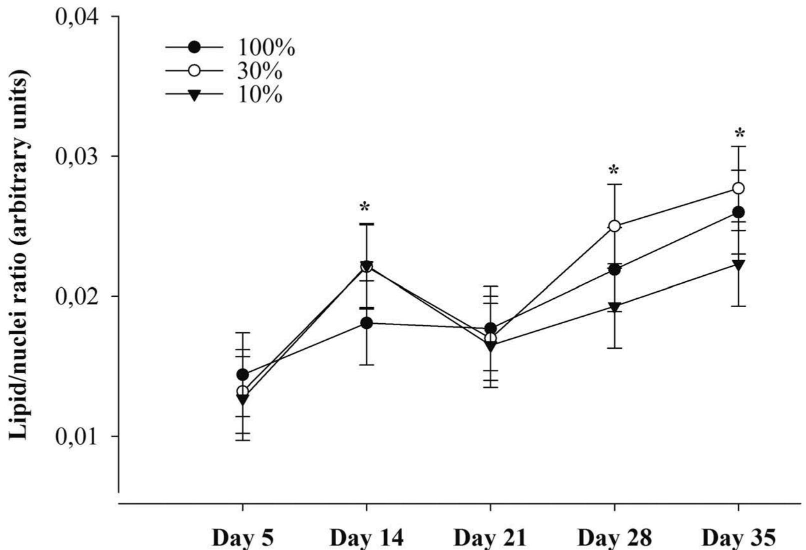


**Figure 2.** Influence of a gradual decrease in the concentrations of insulin, dexamethasone, rosiglitazone, 3-isobutyl-1-methylxanthine (IBMX) and biotin in the induction medium to 30% and 10% of their original concentrations on lipid incorporation by bovine ASC. The '100% medium' contained 10 µg/mL insulin, 1 µM dexamethasone, 20 µM rosiglitazone, 250 µM IBMX and 33 µM biotin in the recipes used by Riedel et al. [22]. The results are presented as means ± SEM of three independent experiments with two replicates. *Asterisks indicate an effect of factor day with $P < 0.05$ to day 5.

by day 28 and day 35 than the '30% medium'. Hence, we concluded that the reduction of induction supplements to 30% of those used by Riedel et al. [23] was the most effective way of achieving adipogenic induction in bovine ASC. We therefore used 3 µg/mL bovine insulin, 0.3 µM dexamethasone, 10 µM rosiglitazone, 100 µM

IBMX and 10 μM biotin for induction in all the following experiments.

### *Experiment 2: in vitro induction and differentiation potential of bovine ASC*

To investigate the differentiation potential of bovine ASC, the cells were kept in induction medium developed in Experiment 1 for 2 d and further transferred into seven different types of differentiation medium, as described in Table 3 for either 7 or 14 d. The differentiation media affected lipid accumulation as shown by Nile red staining (Figure 3). Microscopically, the presence of non-polar lipids could primarily be seen following treatments TRT-2, TRT-3, TRT-5 and TRT-6, i.e. after treatments in which BSL was present; the presence of non-polar lipids was negligible in TRT-1, TRT-4 and the control treatment (TRT-Con). The lipids were visible as early as day 7 in the BSL-treated groups and appeared to become intensified at day 14, especially, in treatments in which FBS was absent (TRT-3 and TRT-6). Of note, the accumulation of non-polar lipids in the BSL-treated groups was greatly dependent on the presence of AsA. Despite a quantitatively similar content of lipids (see below), the pattern of lipid accumulation was mostly (albeit not exclusively) diffuse in the absence of AsA (TRT-2 and TRT-3). In contrast, the presence of AsA (TRT-5 and TRT-6) enhanced the build-up of larger fat vacuoles in defined clusters of adipocytes with other areas appearing devoid of lipids. The formation of the defined adipocyte clusters was linked to a change in the cellular shape of the fat-accumulating cells.

The quantitative statistical evaluation of non-polar lipid build-up in cells (in terms of lipids/nuclei ratio) is presented in Figure 3 graph a. The influence of the various treatments and of the factor day were both statistically significant ($P < 0.001$). Furthermore, a treatment × day interaction ($P = 0.001$) was detected that was based on a significantly enhanced lipids/nuclei ratio in TRT-3 and TRT-6 compared with all other treatments after 14 d of culture.

The incubation of cells in FBS-free medium not only enhanced the development of non-polar lipids in cells, but also inversely affected cell density (Figure 3 graph b). The DAPI fluorescence signal (representing the density of cell nuclei) was significantly affected by treatment ($P < 0.001$) and was lowest in treatments without FBS (TRT-Con, TRT-3 and TRT-6) but highest in the treatment regime with FBS and AsA (TRT-4), treatments TRT-1, TRT-2 and TRT-5 showing intermediate values. Neither day of treatment ($P = 0.586$) nor TRT × day interaction ($P = 0.051$) was significant.

Immunocytochemistry was carried out in order to visualize the presence of cell-specific markers for ASC and adipocytes. Cells at the pre-adipocyte stage were positive for NT5E, THY1, ENG, PDGFRα, DLK1, GLUT4 and FAS (Figures 1 and 4), but FABP4 was absent (Figure 4). A visual increase in the development and intensity of the DLK1 surface marker occurred in the differentiation media (e.g. TRT-Con and TRT-1 to TRT-4). With an increase in culture age and the emergence of adipocytes, however, the DLK1 surface marker became less intense in the cells that were accumulating larger lipid droplets (TRT-5 and TRT-6). Cells with larger lipid droplets also showed a diverse round morphology plus evident FABP4 around the lipid droplets (Figure 4; TRT-5 and TRT-6). Cell surface markers NT5E, THY1, and ENG were present not only at the pre-adipocyte stage, but also during and after the differentiation process of ASC, but with a clear change in the intensity of these markers. The major change was observed in NT5E localization: at first, it was present around the nuclei, but with ageing and differentiation, it was found mainly on the cell surface (Figure 4). Immunostaining for PDGFRα was visible at all stages and found inside cells and on cell boundaries. With the progression of transformation towards adipocytes, the signal decreased. GLUT4 was present already at the preadipocyte stage and its intensity increased with the differentiation towards adipocytes. This increase was particularly evident in the treatments where BSL was present in the medium as shown in Figure 4. FAS was present throughout the experiments (Figure 4); however, a concentration of FAS signal within the Golgi apparatus was evident with the progression of cellular development towards adipocytes, again, especially in groups treated with BSL.

To analyse the effects of the differentiation medium on the phenotypic changes in ASC further, cells were collected for reverse-transcription quantitative PCR analysis after 14 d in the seven different types of differentiation media. ANOVA identified significant changes in the mRNA abundance of the stem cell markers *NTE5, THY1, ENG* ($P < 0.05$). The Dunn's test revealed that the expression of *NT5E* remained unaltered ($P > 0.05$) compared with pre-adipocyte values when FBS was supplemented in the differentiation medium but decreased significantly ($P < 0.05$) in FBS-free treatments (TRT-Con, TRT-3 and TRT-6; Figure 5(a)). The mRNA abundance for *THY1* was or tended to be lower ($P < 0.05$) in TRT-C, TRT-3, and TRT-6 in contrast with all other

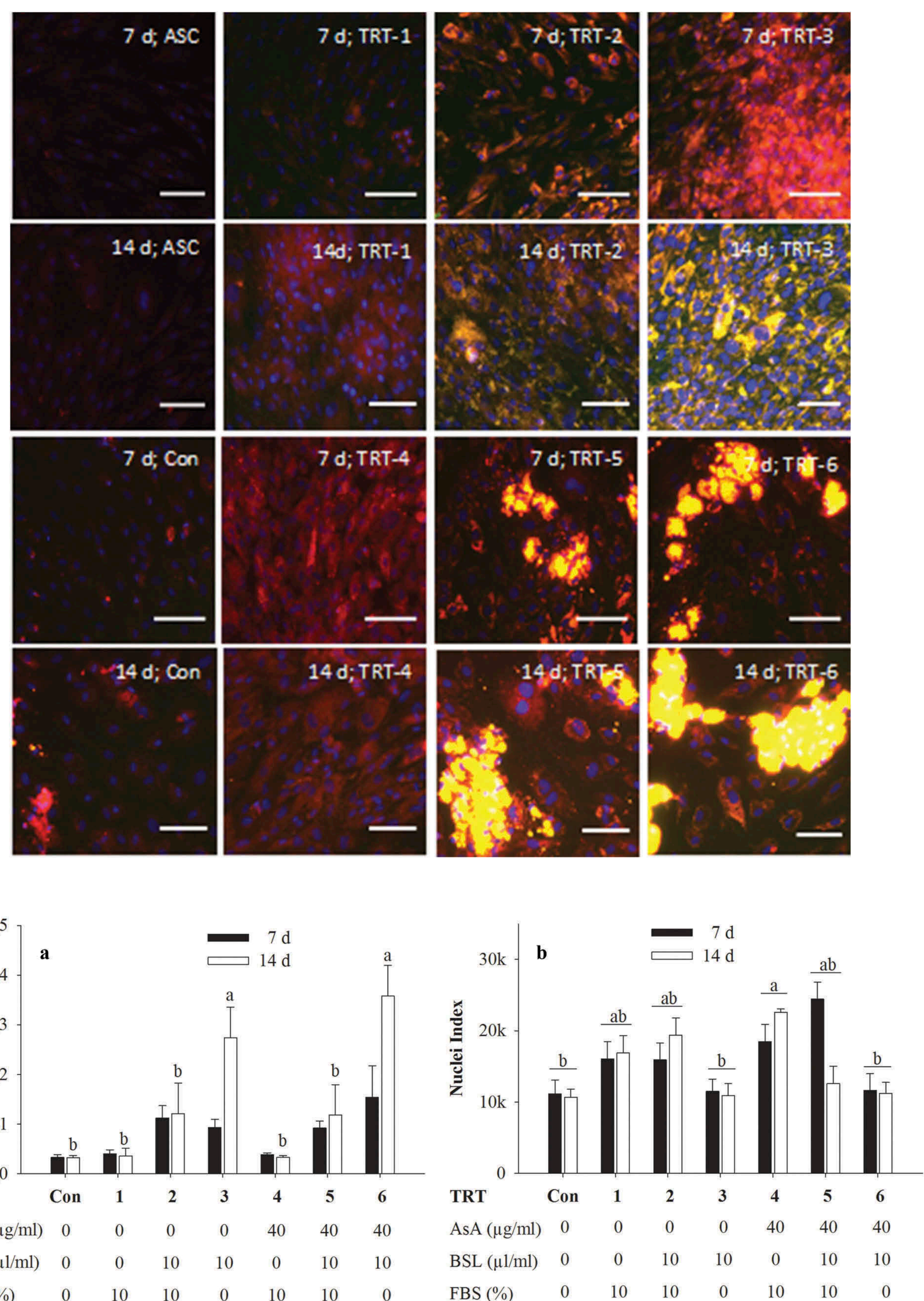


Figure 3. Nile red staining of lipids in adipocytes after 2 d in induction medium followed by 7 d or 14 d of incubation in various adipocyte differentiation media. Nile red was imaged at (excitation/emission) 515 nm/590 nm for total lipids (coded to red) and 475 nm/570 nm (emission) for non-polar lipids (coded to green). The green-red overlay results in bright yellow colour for lipid droplets. Nuclei were stained with DAPI and imaged at 358 nm/461 nm (emission). Scale bar = 100 µm (20× objective). The graph A shows quantification of non-polar lipids (475 nm/570 nm) relative to DAPI fluorescence (358 nm/461 nm) for three independent experiments with two replicates while graph B shows corresponding DAPI fluorescence. The data symbolize means ± SEM. [a,b]Mean values with different superscripts are significantly different ($P < 0.05$). TRT, treatment; Con, negative control; AsA, ascorbic acid; BSL, bovine serum lipids; FBS, foetal bovine serum.

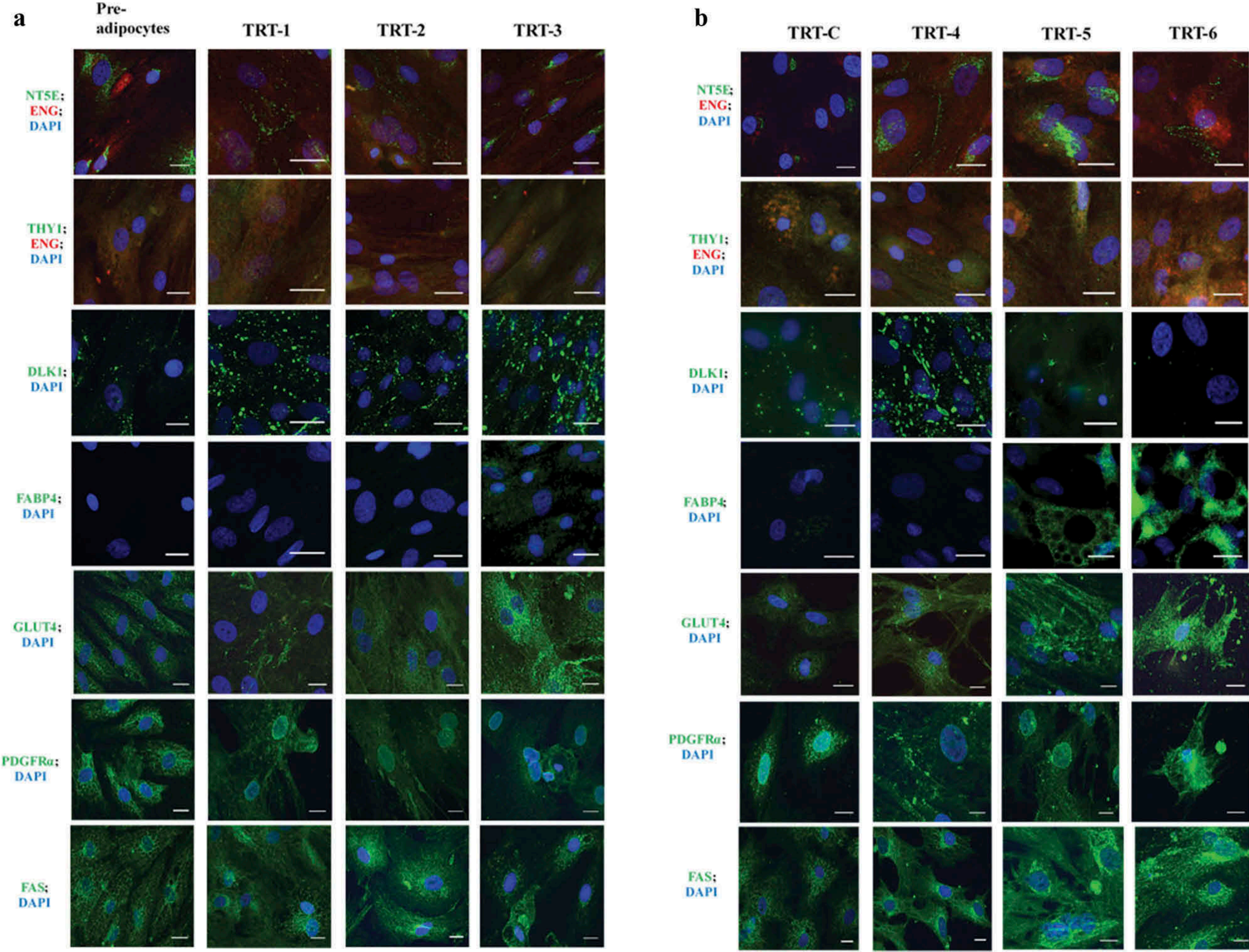


**Figure 4.** Immunocytochemical classification of bovine subcutaneous adipose-derived stem cells at different stages of development analysed for the presence of different ASC and adipocyte markers. All images were made after 14 d in different types of differentiation media (TRT-1 through TRT-6; for code see Table 3). The colour code of the antigen labels represents the fluorophore colour of the imaged antigen (red or green) and nuclear stain (DAPI, blue), respectively. Scale bar = 25 μm (63 × objective).

treatments (Figure 5(b)). The expression of *ENG* decreased or tended to decrease in all treatments compared with the pre-adipocyte stage with decreases being most pronounced in the FBS-free groups (TRT-Con, TRT-3 and TRT-6) and treatments with AsA (TRT-3 to TRT-5; $P < 0.05$; Figure 5(c)). Analysis of *PDGFRα* did not reveal any significant changes (Figure 5(d)).

The expression of the mature adipocyte marker *FABP4* increased when media were supplemented with BSL in the absence of FBS (TRT-3 and TRT-6, $P < 0.05$), however, the increase was two-fold higher in the additional presence of AsA (TRT-6, $P < 0.05$). In the presence of AsA, an increase in FABP4 expression was also seen when medium contained BSL and FBS (TRT-5; Figure 5(e)). The other mature adipocyte markers (*LPL*, *GLUT4*, and *PPARγ*) showed a behaviour similar to *FABP4*; however, due to high variance among animals, these changes were not significant (Figure 5(f–h)).

### *Experiment 3: effect of AcA, glucose and various coating on adipogenesis*

Based on the results of Experiment 2, we used a differentiation medium containing BSL and AsA but no FBS to test the hypothesis that the concentration of AcA in the medium has an effect on non-polar lipid accumulation, and that this effect is influenced by the availability of glucose in the culture medium. However, as the factor glucose was not significant, it was removed from the statistical analysis to enable three-way ANOVA with the factors AcA, day and coating, the last mentioned being used to test whether different coatings affect cell attachment/viability. None of the

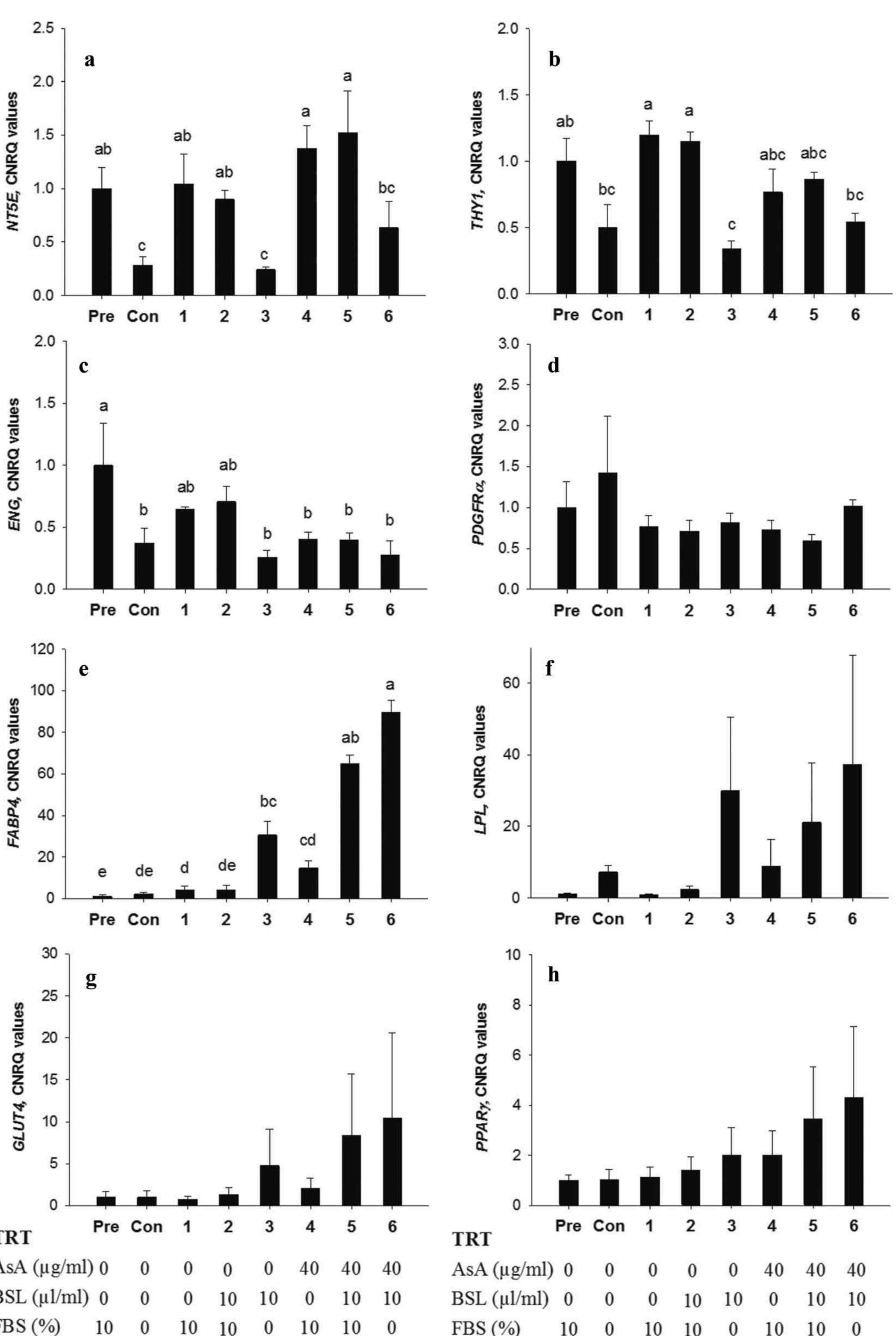


**Figure 5.** Relative mRNA expression for *NT5E, THY1, ENG, PDGFRa, FABP4, LPL, GLUT4 and PPARγ* in bovine subcutaneous ASC after *in vitro* transdifferentiation. Quantitative reverse transcription PCR analysis of *NT5E* (A), *THY1* (B), *ENG* (C), *PDGFRa* (D), *FABP4* (E), *LPL* (F), *GLUT4* (G) and *PPARγ* (H) are given as the mean ± SEM of four animals with three replicates. Pre, pre-adipocyte (RNA extracted before induction); Con, negative control; TRT = treatment; FBS, foetal bovine serum; AsA, ascorbic acid; BSL, bovine serum lipids. Letters (a-e) are used to denote statistical difference; columns within one graph are different if they do not share a common letter ($P < 0.05$).

**Table 4.** Cell nuclei index and lipid/nuclei ratio of bovine ASC kept for 7 or 14 d intervals in plastic ware with various coatings and differentiation media containing various concentrations of acetic acid (AcA).

| Nuclei index | | | | | | |
|---|---|---|---|---|---|---|
| Factor[a] | | | | | SEM | P-value |
| Coating[b] | None | poly-L-lysine | Collagen-A | Gelatin-A | | |
| | 11724[B] | 12846[A] | 11767[B] | 12612[A] | 242 | < 0.001 |
| AcA | 0 mM | 10 mM | 20 mM | | | |
| | 12736[A] | 12411[A] | 11565[B] | | 192 | < 0.001 |
| Day | 7 d | 14 d | | | | |
| | 12892[A] | 11583[B] | | | 157 | < 0.001 |
| **Lipid/Nuclei ratio** | | | | | | |
| Factor | | | | | SEM | P-value |
| Coating | None | poly-L-lysine | Collagen-A | Gelatin-A | | |
| | 0.035 | 0.029 | 0.035 | 0.033 | 0.004 | 0.741 |
| AcA | 0 mM | 10 mM | 20 mM | | | |
| | 0.029 | 0.034 | 0.036 | | 0.004 | 0.438 |
| Day | 7 d | 14 d | | | | |
| | 0.026[B] | 0.041[A] | | | 0.003 | < 0.001 |

Non-polar lipids were measured after Nile red staining (excitation/emission, 475 nm/570 nm), whereas nuclei were assessed by DAPI fluorescence (excitation/emission, 358 nm/461 nm).
[a]Factor glucose was removed from the analyses after verification that the different glucose concentrations had no significant effect.
[A,B]Values with similar capital letters in a single row do not differ statistically at $P < 0.05$.

two- and three-way interactions among these factors were significant, and they are therefore not presented.

The factor coating affected the nuclei index ($P < 0.001$), which is indicative of cell density, with decreased values in non-coated and collagen-A-coated wells compared with poly-L-lysine or gelatin-A-coated materials (Table 4). Factor AcA also influenced the nuclei index ($P < 0.001$), with decreased values at 20 mM AcA compared with 0 and 10 mM AcA (Table 4). The nuclei index further changed with regard to factor day of treatment ($P < 0.001$), with slightly lower values after 14 d in differentiation medium as compared with 7 d (Table 4).

The lipid/nuclei ratio, indicative of the accumulation of lipid droplets, was only affected by the factor day of treatment ($P < 0.001$), with higher values after 14 d in the differentiation medium as compared with 7 d (Table 4).

## Discussion

Adult adipose stem cells are an important source of MSC and can be conveniently recovered and efficiently used in regenerative therapies because of their immune-compatible properties. In many species, ASC have been isolated and studied for their multi-linage differentiation, e.g. in man [25], cattle [20] and mouse [26]. The removal of pre-adipocytes from adipose depots is an easy cost-effective process and does not require co-incubation with toxic chemicals for cellular differentiation. In preliminary experiments, we found that ASC are especially abundant in younger animals. Therefore, we used calves of < 11 months of age for the explant cultures, adipose tissue was isolated caudally to the mandible, in the region of the 2nd to 3rd vertebrae, immediately after conventional slaughter. The harvested ASC had a fibroblast-like shape when they were in the growth phase but became round to oval when transdifferentiating into functional adipocytes. The differentiation and shape of cells depends on many important factors such as the initial plating concentration of cells [27] and the commitment of cells towards adipogenesis through the increased expression of the *DLK1, FABP4* and *PPARγ* genes. The use of an induction media cocktail seems to be necessary, especially for the activation of *PPARγ* and its downstream genes. PPARγ is a member of the nuclear steroid receptor family and is activated when bound to natural long chain fatty acids. Previous studies carried out on primary ASC have shown an increase in the mRNA of *PPARγ* during the process of differentiation [28]. Although the increase in the mRNA of *PPARγ* was not significant in the present study, the treatments that accumulated lipids most intensely, at least, appeared to have a higher expression of *PPARγ*, especially treatments receiving BSL and AsA.

Substances that activate PPARγ (e.g. thiazolidinedione and rosiglitazone) act as potent stimulators of ASC differentiation [29]. The presence of dexamethasone in the induction medium stimulates the expression of *PPARγ* and other nuclear factors, and ASC can progress towards transdifferentiation [30]. However, the use of dexamethasone for extended periods of time and in excessive amounts can be detrimental for ASC induction and differentiation [31]. Moreover, insulin has a well-known adipogenic effect and is therefore often used to activate various metabolic genes that finally trigger adipogenesis, including *PPARγ* [31].

Hyperinsulinic concentrations in the culture medium are therefore helpful to induce transdifferentiation.

Published protocols for the induction of adipogenic transdifferentiation regularly use excessively high concentrations of PPARγ agonists, dexamethasone and insulin, together with IBMX and biotin. To elucidate whether such elevated concentrations are too high for bovine ASC and induce negative effects on adipogenic transdifferentiation, we first performed a titration experiment in which we reduced the concentration of such supplements to 30% and 10% of their initial values. Our results demonstrate no substantial difference in the induction of transdifferentiation among the three concentrations tested. Since the 30% medium performed numerically (although not statistically) best over a prolonged period of time, we used and recommend this intermediate concentration of supplements for further experiments.

We further aimed at characterizing the marker proteins of ASC in Experiment 2. We had previously reported the immunocytochemical characterization of bovine subcutaneous ASC [20]. In that previous study, we focused on the prominent effects that BSL elicit on adipocyte differentiation and on the changes of cellular markers during that process. In the present investigation, we additionally used the quantification of Nile red fluorescence to measure lipid accumulation. This approach verified that the addition of BSL without FBS in the differentiation medium significantly improved the accumulation of non-polar lipids in cells, suggesting that serum-free medium and BSL enhanced the adipogenic potential of ASC. Bovine serum lipids are known to augment the internalization of long chain fatty acids in the presence of insulin via FABP4 [32]. The internalized fatty acids can then increase adipogenesis by acting as natural ligands for PPARγ and by serving as a direct fatty acid source for triglyceride synthesis.

Among the important mesenchymal stem cell markers that change during adipogenesis, the expression of ENG depends on the stem cell source (bone marrow or adipose tissue) and the period of maintenance in the culture medium [33]. As an extension of our previously reported results, the present study revealed a higher amount of ENG at pre-adipocyte stage, evidenced both by immunocytochemistry and by quantitative reverse transcription PCR. In addition to ENG, the cultured pre-adipocytes stained positively for NT5E and THY1. These results are in agreement with the results of Jones et al. who further demonstrated that the presence of ENG, NT5E and THY1 occurs irrespective of their passage number in culture [34]. In human mesenchymal stem cells (MSC), the positive expression of ENG, NT5E and THY1 is taken as evidence for the pluripotency of stem cells [35]; however, Boxall and Jones [36] have noted the absence of THY1 in MSC of goats and sheep. Our results showed the noticeable expression of NT5E, THY1, and ENG protein even at later stages of bovine subcutaneous ASC differentiation to adipocytes. This similarly applied to *PDGFRα* where immunocytochemical experiments showed an only mild decrease in signal strength during differentiation with no changes in mRNA abundance. Therefore, the use of these markers to identify cells as being potent for lineage differentiation is debatable. In agreement with this suggestion, Mark et al. [37] have reported that only 15% of human bone marrow cells have the potential to differentiate towards the adipogenic lineage rather than osteogenic lineage, as based on the presence or absence of ENG.

The omission of FBS from the incubation medium in the present study decreased the nuclei index, i.e. stopped cell proliferation, and was associated with the lower mRNA expression of *NT5E* and *ENG*. However, cells undergoing the FBS-free treatments (TRT-Con, TRT-3 and TRT-6) still had clearly detectable mRNA and protein levels for NT5E and ENG. Therefore, the expression of the two markers should not be linearly associated with MSC renewal or MSC renewal capability. The changes in the mRNA expression of *THY1* largely mirrored those of *NT5E* and *ENG*. This supports recent findings that *THY1* expression decreases with the differentiation of 3T3-L1 cells into functional adipocytes [38], and that knockout of *THY1* predisposes mice to adiposity [38,39].

In contrast to the ASC marker genes, the mRNA expression of *FABP4* was induced by the absence of FBS and in the presence of BSL in the differentiation medium. A striking finding was that the supplementation with AsA greatly enhanced the expression of *FABP4*. The latter was even true for media containing FBS (TRT-5) or being devoid of BSL (TRT-4). The combination of BSL and AsA (in the absence of FBS; i.e. TRT-6) was most effective in inducing the mRNA expression of *FABP4*. Cuaranta-Monroy et al. [40] have previously demonstrated the positive effect of AsA on the expression of *FABP4* in adipocytes differentiated from mouse embryonic stem cells. Our study extends these findings by demonstrating that the increased expression of *FABP4* is linked to the greater lipid accumulation in selected *FABP4*-containing cell clusters but not necessarily the greater lipid accumulation by the whole cell culture. Such clusters were typical for TRT-5 and TRT-6, which exhibited the highest expression of *FABP4* and visually contained many functionally mature adipocyte clusters.

The cellular function of FABP4 is the coupling of fatty acids to several molecular targets as a fatty acids chaperone [41]. Via peroxisome proliferator response elements, the transcription of *FABP4* is directly coupled to PPARγ and is thus prominently induced during ASC

differentiation [42]. Vice versa, FABP4 appears to be a negative feed-back master regulator of PPARγ and plays a crucial role in the ubiquitination of PPARγ and, consequently, the down-regulation of insulin sensitivity in the mature adipocyte. The latter is seen as a major cause of morbidities related to insulin resistance [43]. Thus, the present study leads to the valuable conclusion that adipocyte differentiation and the expression of *FABP4* are regulated by AsA independently of the global lipid accumulation of an ASC culture.

Ono et al. [44] have reported that AsA stimulates the synthesis of procollagen and accelerates the differentiation of adipocytes, the latter being evidenced by the development of larger intracellular lipid droplets. Our results confirm that AsA has indeed a synergistic effect in the transformation of cells, although our results demonstrate that BSL is the most important supplement for the accumulation of non-polar lipids in bovine adipocytes, whereas AsA does not have a significant influence on the quantity of intracellular non-polar lipids, when BSL is present. To our knowledge, this is the first study reporting the dissimilar role of AsA and BSL for the amplification of *FABP4* with synergistic effects on adipogenic differentiation.

The antioxidant capacity of AsA might play a central role in this process. An earlier study by Lee et al. [45] has demonstrated that a plant-derived antioxidant (puerarin) also enhances differentiation and the upregulation of the expression of *FABP4* in 3T3-L1 cells. Furthermore, the knockout of *FABP4* decreases oxidative stress and uncouples obesity from inflammation in macrophages [46]. Together with the findings of the present study, these results support the conclusion that FABP4 plays a central role in the redox status of the cell; this is not entirely explainable by the degree of lipid accumulation. Vice versa, the redox status has a negative feedback on the expression of *FABP4* itself.

The treatment conditions leading to the largest amounts of FABP4 had the lowest abundance of DLK1 protein. This agrees with the known role of DLK1 as a negative regulator of adipocytes differentiation [47]. DLK1 is known to bind to fibronectin in the extracellular matrix; the fibronectin-DLK1 complex then interacts with integrin receptors to inhibit adipogenesis via the mitogen-activated protein kinase/extracellular-signal regulated kinases (MEK/ERK) pathway [48].

In addition to *FABP4*, we also analysed gene expression of other functionally relevant genes of mature adipocytes, namely, glucose transporter type 4 (*GLUT4*) and of lipoprotein lipase (*LPL*). *GLUT4* mediates the insulin-stimulated uptake of glucose into adipocytes and skeletal muscles [49]. Immunocytochemical staining confirmed the presence of GLUT4 in pre-adipocytes and all other treatment conditions with increasing intensity in treatment conditions that favoured the development of mature adipocytes (TRT-5 and TRT-6). Also quantitative real-time PCR indicated that highest values are associated with these two treatment conditions. However, in contrast to *FABP4*, we detected high variability in the expression levels between the individual animals. The same was true for the expression of *LPL* for which the expression pattern was again very similar to *FABP4*. Unfortunately, we could not identify a suitable antibody recognizing bovine LPL by immunocytochemistry. We therefore decided to investigate protein expression of fatty acid synthase (FAS), another enzyme with prominent role in triglyceride accumulation. FAS expression was identified under all treatment conditions with strongest staining in culture media that contained AsA as well as BSL (TRT-5 and TRT-6) that promoted the differentiation of ASC to mature adipocytes most effectively.

A special feature of bovine adipocyte metabolism *in vivo* is that AcA rather than glucose is the principal source of fatty acid synthesis [19]. Therefore, we tested the effects of various glucose and AcA concentrations on ASC differentiation in Experiment 3. With regard to the conventional glucose concentration in cell culture media, namely 25 mM, our results showed that a lowering of the glucose concentration to 10 mM did not have any significant effect on ASC lipid incorporation at various levels of AcA. Increasing the concentration of AcA to 10 and 20 mM induced no significant effects of AcA on lipid storage although lipid accumulation was numerically higher. All in all, this means that a glucose concentration of 10 mM does not limit the adipogenic transdifferentiation of bovine ASC and that AcA may have a stimulating effect on this process that requires further investigation. Nonetheless, an AcA concentration of 20 mM can lead to a decrease in nuclei density, i.e. cell number, and may thus be potentially too high and elicit detrimental effects on the cultured cells.

A valuable methodological result of Experiment 3 was that the coating of the culture dishes with poly-L-lysine and gelatin-A was associated with higher nuclei indices. This increase can be taken as an indication that cell survival and adherence improve if the cells are grown in the presence of these coatings. Experiment 3 also verified an increase in the nuclei ratio between 7 and 14 d, as similarly observed in BSL-containing, ASC-containing and FBS-free medium in Experiment 2, and thus identifies such a medium composition as highly suitable for the differentiation of bovine ASC-derived adipocytes.

In conclusion, this study shows that the differentiation and adipogenesis of bovine subcutaneous ASC-derived adipocytes is optimal in poly-L-lysine or gelatin-A coated culture wells, in the absence of FBS but in the presence of

BSL and AsA. The last mentioned appears to have a unique role in *FABP4* induction, although this function is not linearly related to the primarily BSL-driven lipid accumulation and is more likely to be associated with the redox properties of AsA. The study further indicates that AcA may stimulate lipid accumulation in ACS-derived adipocyte cultures. However, the latter effect may be difficult to uncover in the presence of BSL and glucose as alternative fuels for adipogenesis and therefore requires further investigation.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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# Chapter 6: The combined influence of magnesium and insulin on central metabolic functions and expression of genes involved in magnesium homeostasis of cultured bovine adipocytes

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*Article*

# The Combined Influence of Magnesium and Insulin on Central Metabolic Functions and Expression of Genes Involved in Magnesium Homeostasis of Cultured Bovine Adipocytes

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**Abstract:** At the onset of lactation, dairy cows suffer from insulin resistance, insulin deficiency or both, similar to human diabetes, resulting in lipolysis, ketosis and fatty liver. This work explored the combined effects of different levels of magnesium (0.1, 0.3, 1 and 3 mM) and insulin (25, 250 and 25,000 pM) on metabolic pathways and the expression of magnesium-responsive genes in a bovine adipocyte model. Magnesium starvation (0.1 mM) and low insulin (25 pM) independently decreased or tended to decrease the accumulation of non-polar lipids and uptake of the glucose analog 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG). Activity of glycerol 3-phosphate dehydrogenase (GPDH) was highest at 25 pM insulin and 3 mM magnesium. Expression of *SLC41A1* and *SLC41A3* was reduced at 0.1 mM magnesium either across insulin concentrations (*SLC41A1*) or at 250 pM insulin (*SLC41A3*). *MAGT1* expression was reduced at 3 mM magnesium. *NIPA1* expression was reduced at 3 mM and 0.1 mM magnesium at 25 and 250 pM insulin, respectively. Expression of *SLC41A2*, *CNNM2*, *TRPM6* and *TRPM7* was not affected. We conclude that magnesium promotes lipogenesis in adipocytes and inversely regulates the transcription of genes that increase vs. decrease cytosolic magnesium concentration. The induction of GAPDH activity by surplus magnesium at low insulin concentration can counteract excessive lipomobilization.

**Keywords:** magnesium; insulin; lipomobilization; adipocytes; cattle; adipose tissue; ketosis; fatty liver; magnesium-responsive genes; glycerol-3-phosphate dehydrogenase

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## 1. Introduction

During the transition period, i.e., three weeks prepartum to three weeks postpartum, dairy cows undergo a dramatic change in their physiological state due to the onset of lactation. At the onset of lactation, the energy demand increases abruptly and cannot be met by adequate intake of feed dry matter [1]. The consequence is a negative energy balance. To compensate the inadequate energy intake, the balance between lipogenesis and lipolysis in adipose tissue is shifted towards dominant lipolysis. This state of lipomobilization is linked to very low serum insulin concentrations, insulin resistance or both [2]. The decreased insulin sensitivity of adipose tissue and skeletal muscles [3] reduces glucose uptake by these tissues and consequently more energy metabolites, particularly glucose, are available for the mammary gland [4]. The reduction of insulin signals is physiological and necessary; however, any exaggeration predisposes the animals to metabolic diseases such as ketosis, fatty liver, milk fever, metritis, retained placenta or displaced abomasum [5,6].

Consequences are high veterinary costs [7] and losses in milk production and reproductive performance [8].

Previous experimental findings point to the fact that marginal Mg availability may promote ketosis in periparturient cows [9]. In contrast, dietary Mg provision far above requirement (0.61% of dry matter) increased fat-corrected milk production postpartum [10]. Studies in ewes with Mg supplementation above requirement suggest that such effects may originate from improved regulation of energy metabolism as demonstrated by decreased non-esterified fatty acids (NEFA) [11] and stabilized glucose concentrations in blood [12]. In contrast, experimentally induced hypomagnesaemia promoted lipolysis [13] and decreased both insulin responsiveness and insulin-mediated glucose disposal in sheep [14].

Magnesium interacts with numerous enzymes and substrates and thus plays a prominent role in energy metabolism, glucose homeostasis [15] and the regulation of triglyceride concentration in blood [16]. In this regard, a reciprocal influence of insulin and magnesium is particularly important. Magnesium is essential for phosphorylation of the insulin receptor and for the activity of downstream kinases. Adequate intracellular Mg concentrations are therefore essential for efficient insulin signaling [17]. At the same time, insulin promotes the retention of Mg in cells by reducing the activity of the main cellular magnesium extrusion system SLC41A1, a $Na^{+}/Mg^{2+}$ exchanger [18].

Insulin is thus a magnesiotropic hormone and, in turn, magnesium is an amplifier of the insulin signal. Consequently, adequate magnesium supply can increase the sensitivity towards insulin and has thus potential to alleviate exaggerated lipomobilization. The present research aimed to prove the hypothesis that supplementation of magnesium above physiological levels could improve insulin sensitivity of adipocytes and thereby reduce lipomobilization [19]. If valid, this could open new therapeutic options to prevent or alleviate metabolic diseases in dairy cows.

## 2. Results

### *2.1. Effect of Insulin and Mg on the Content of Non-Polar Lipids (Lipid/Nuclei Ratio)*

To assess the combined influence of insulin and magnesium on cellular lipid content, the accumulation of intracellular non-polar lipids was measured by Nile-Red staining of adipocytes from six animals after 14 and 21 days in differentiation medium. To correct variations in cell density, the means of fluorescence signals for Nile red (lipid index) were divided by the means of DAPI fluorescence (nuclei index), yielding the lipid/nuclei ratio. Because of failed normality test, data were log-transformed before statistical evaluation.

After 14 days of incubation in differentiation medium (Figure 1A), log-transformed lipid/nuclei ratio was affected by the factors insulin ($P = 0.005$) and Mg ($P = 0.009$) with a trend for Mg $\times$ insulin interaction ($P = 0.078$). The lowest insulin concentration (25 pM; LSM = 1.45 $\pm$ 0.051) showed the least accumulation of non-polar lipids per cell in comparison to a high physiological (250 pM; LSM = 1.67 $\pm$ 0.051) and a supraphysiological insulin concentration (25,000 pM; LSM = 1.76 $\pm$ 0.051), evidenced by a lower log-transformed lipid/nuclei ratio at 25 pM insulin ($P \leq 0.05$ each). Across insulin concentrations, a significantly lower accumulation of non-polar lipids was observed at the lowest Mg concentration of 0.1 mM (LSM = 1.43 $\pm$ 0.056) in comparison to 0.3 mM (LSM = 1.67 $\pm$ 0.056), 1 mM (LSM = 1.71 $\pm$ 0.056) and 3 mM Mg (LSM = 1.70 $\pm$ 0.056) in the medium ($P \leq 0.05$ each), where 1 mM was chosen to represent physiological Mg conditions [20].

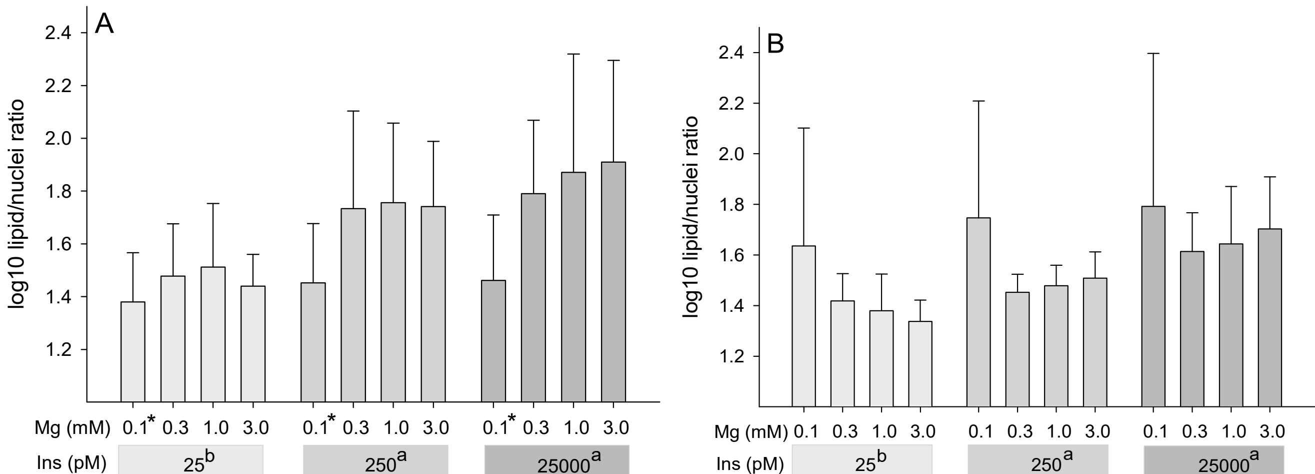


**Figure 1.** Quantification of non-polar lipids (ex. 475 nm/em. 570 nm) after 14 days (**A**) and after 21 days (**B**) in various differentiation media containing different concentrations of magnesium (Mg) or insulin (Ins). Non-polar lipids are presented relative to dsDNA signal (DAPI fluorescence of nuclei ex.358 nm/em. 461 nm). Results are given as means ± SD of six animals with two replicates per animal. * Asterisks indicate smaller LSM at 0.1 mM Mg compared to all other Mg concentrations ($P \leq 0.05$). [a,b] Different superscript letters indicate different LSM for the factor insulin ($P \leq 0.05$).

After 21 days of culture (Figure 1B), the log-transformed lipid/nuclei ratio was affected by the factor insulin only ($P = 0.001$). A supraphysiological insulin concentration (25,000 pM; LSM = 1.69 ± 0.034) showed the highest log-transformed lipid/nuclei ratio in comparison to a high physiological (250 pM; LSM = 1.55 ± 0.034) and the low (25 pM; LSM = 1.44 ± 0.034) insulin concentration ($P \leq 0.05$ each). An insulin concentration of 25 pM also tended to have lower log-transformed lipid/nuclei ratio compared to 250 pM ($P = 0.055$). No effects of Mg ($P = 0.34$) or Mg × insulin interaction ($P = 0.20$) were observed after 21 days in the differentiation medium. Representative images of lipid accumulation after 14 and 21 days at 1 mM Mg and different insulin concentrations are shown in Figure 2.

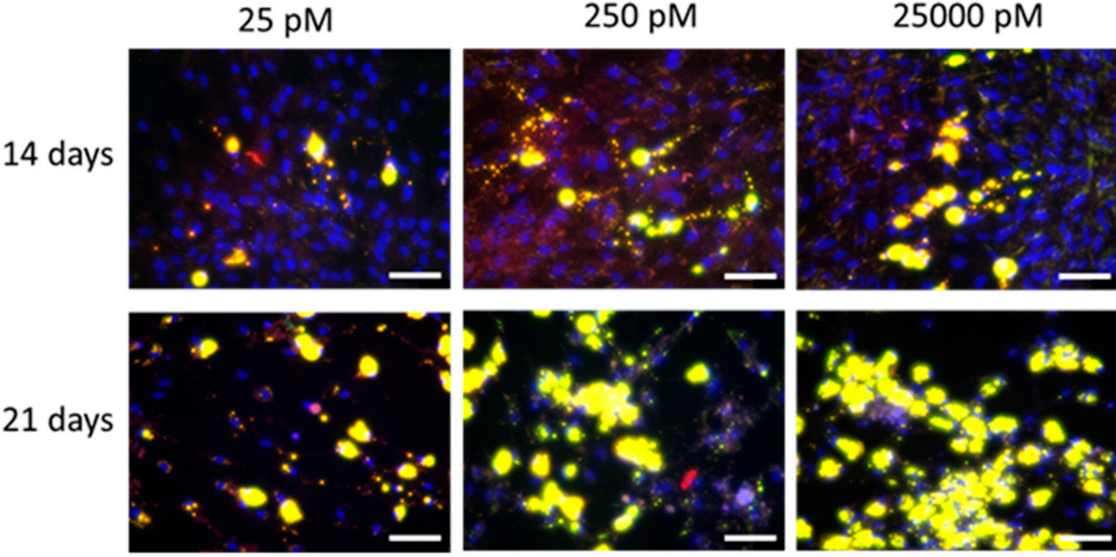


**Figure 2.** Representative Nile red staining of intracellular lipids in adipocytes after 14 days and 21 days of incubation using various insulin concentrations (25 pM, 250 pM and 25,000 pM) at a physiological magnesium concentration (1 mM). Nile red was imaged for total lipids at 515 nm/590 nm (ex./em.) and coded to red. Non-polar lipids were imaged at 475 nm/570 nm (ex./em.) and coded to green. A green-red overlay results in yellow color for lipid droplets. The blue color represents dsDNA of nuclei stained with DAPI and imaged at 358 nm/461 nm (ex./em.). Scale bar = 100 μm (20× objective).

### 2.2. Influence of Insulin and Magnesium on Glucose Uptake of Adipocytes (Glucose/Nuclei Ratio)

The capacity for glucose uptake was examined using a 6-NBDG assay with cells from six animals after 14 and 21 days in differentiation medium. The fluorescence of 6-NBDG (glucose index) was normalized to DAPI fluorescence (nuclei index) as glucose/nuclei ratio in order to assess the capacity of glucose uptake per cell. Because of failed normality test, data were log-transformed before statistical evaluation.

After incubation for 14 days in differentiation medium, log-transformed glucose/nuclei ratio showed a rising trend with higher insulin concentrations ($P = 0.057$) but no effect of Mg concentration ($P = 0.27$) and no Mg × insulin interaction ($P = 0.20$; Figure 3A).

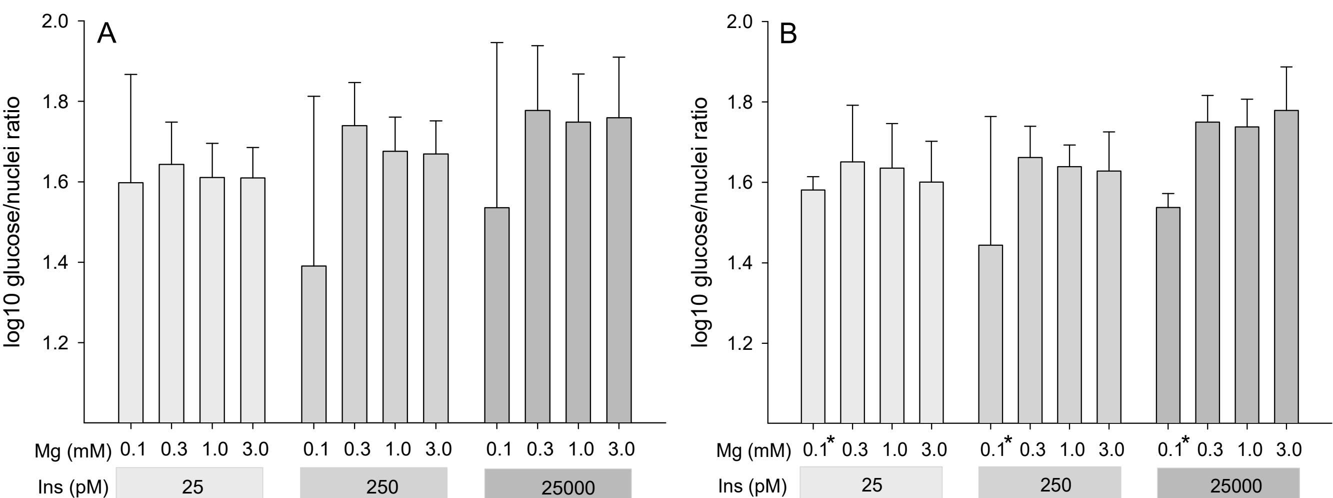


**Figure 3.** Assessment of the capacity for glucose uptake using the fluorescent glucose analog 6-NBDG (ex. 485 nm/em. 530 nm) after 14 days (**A**) and after 21 days (**B**) in various differentiation media containing different concentrations of magnesium (Mg) or insulin (Ins). DAPI fluorescence (ex. 358 nm/em. 461 nm) was used for normalization and results are expressed as log-transformed glucose/nuclei ratio. Results are means ± SD of six animals with two replicates per animal. * Asterisks indicate smaller LSM at 0.1 mM Mg compared to all other Mg concentrations ($P \leq 0.05$).

After 21 days of cultivation, the trend for effect of insulin concentration persisted ($P = 0.10$); however, log-transformed glucose/nuclei ratio was now significantly affected by the factor Mg ($P = 0.007$) with no Mg × insulin interaction ($P = 0.25$). The lowest tested Mg concentration (0.1 mM) had the lowest log-transformed glucose/nuclei ratio in comparison to all other tested Mg concentrations ($P \leq 0.05$ each; Figure 3B).

### 2.3. Influence of Insulin and Magnesium on GPDH Activity of Adipocytes

Glycerol 3-phosphate dehydrogenase (GPDH) activity was investigated according to the manufacturer's instructions with $1 \times 10^6$ cells after 7 days of cultivation in various differentiation media. Least square means of five animals were statistically analyzed. The incubation at various insulin and Mg concentrations showed a statistically significant effect of insulin ($P = 0.045$) and Mg ($P = 0.037$) on the GPDH activity with no Mg × insulin interaction ($P = 0.38$; Table 1). The highest GPDH activity was measured at the lowest insulin concentration (25 pM) with a trend to decrease towards a higher physiological insulin concentration of 250 pM ($P = 0.054$). Cells incubated at the highest Mg concentration (3 mM) tended to have higher activity of GPDH compared to all lower Mg concentrations (0.1 mM, $P = 0.052$; 0.3 mM, $P = 0.062$ and 1 mM, $P = 0.056$), as seen in Table 1.

**Table 1.** Least square means (LSM) of the glycerol-3-phosphate dehydrogenase (GPDH) activity in bovine adipocytes from five animals at various insulin (Ins in pM) and magnesium (Mg in mM) concentrations after 7 days of incubation.

| Ins/Mg | 0.1 MM | 0.3 MM | 1 MM | 3 MM | LSM | SEM | |
|---|---|---|---|---|---|---|---|
| 25 PM | 1.58 | 1.45 | 1.26 | 3.24 | 1.88 | 0.170 | Factor insulin, *P* = 0.045 |
| 250 PM | 1.01 | 1.12 | 1.35 | 1.52 | 1.25 | 0.129 | |
| 25,000 PM | 1.55 | 1.49 | 1.48 | 1.67 | 1.55 | 0.129 | |
| LSM | 1.38 | 1.35 | 1.36 | 2.14 | | | Mg × insulin, *P* = 0.38 |
| SEM | 0.151 | 0.151 | 0.151 | 0.214 | | | |
| | Factor Mg, *P* = 0.037 | | | | | | |

SEM is the pooled standard error of mean. Despite significant effects of insulin and Mg, Holm-Sidak post-hoc test could only identify trends towards highest GPDH activity at the lowest insulin and highest Mg concentration ($P \leq 0.1$).

### 2.4. Influence of Insulin and Magnesium on the Expression of Magnesium-Responsive Genes

The log10-fold changes of the calibrated normalized relative quantity (CNRQ) of Mg-responsive genes of six animals were analyzed after 7 days of incubation in differentiation medium. No changes were found in the expression of the solute carrier family 41 member 2 (*SLC41A2*; Figure 4B), cyclin and CBS domain divalent metal cation transport mediator 2 (*CNNM2*; Figure 4G) and transient receptor potential cation channel subfamily M members 6 (*TRPM6*; Figure 4E) and 7 (*TRPM7*; Figure 4F).

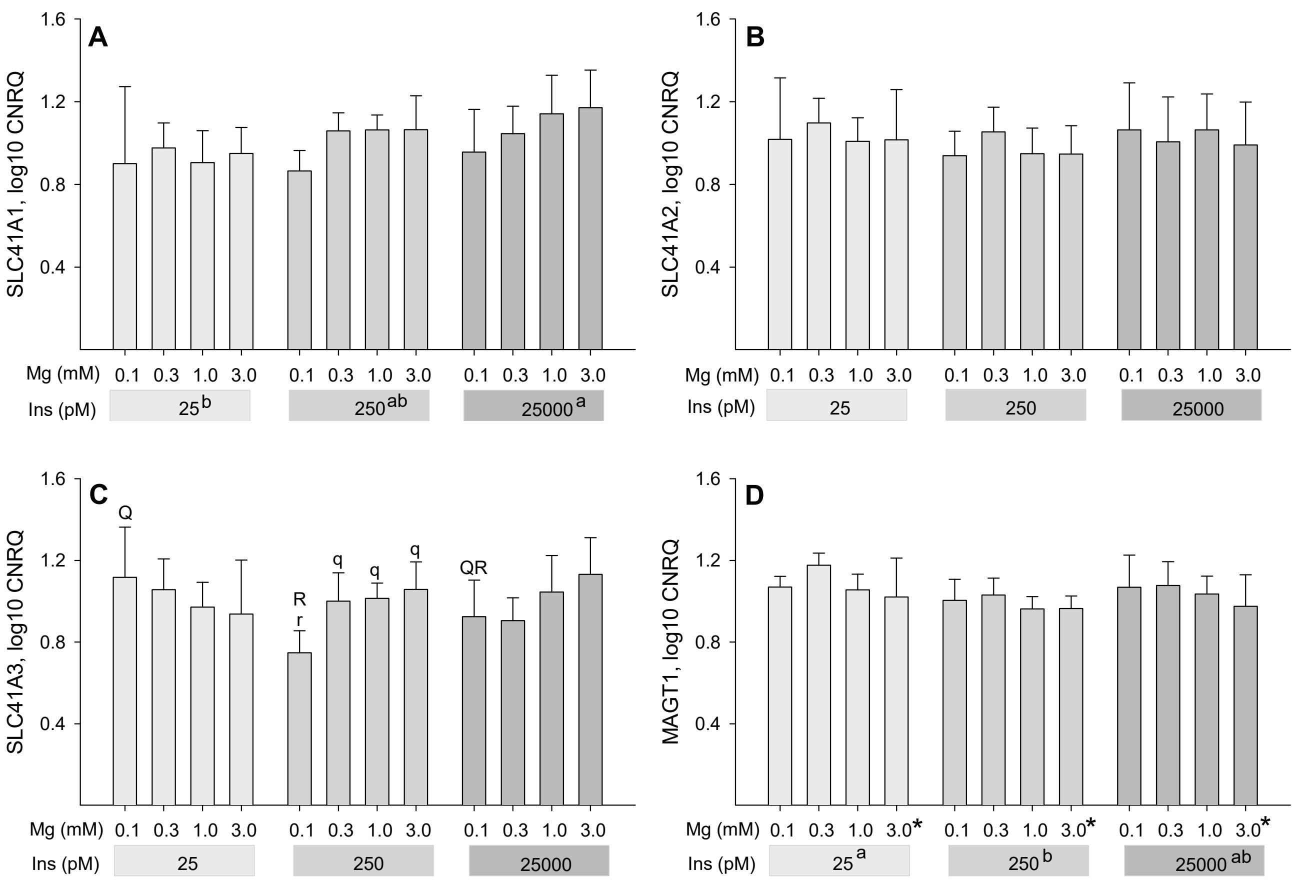


**Figure 4.** *Cont.*

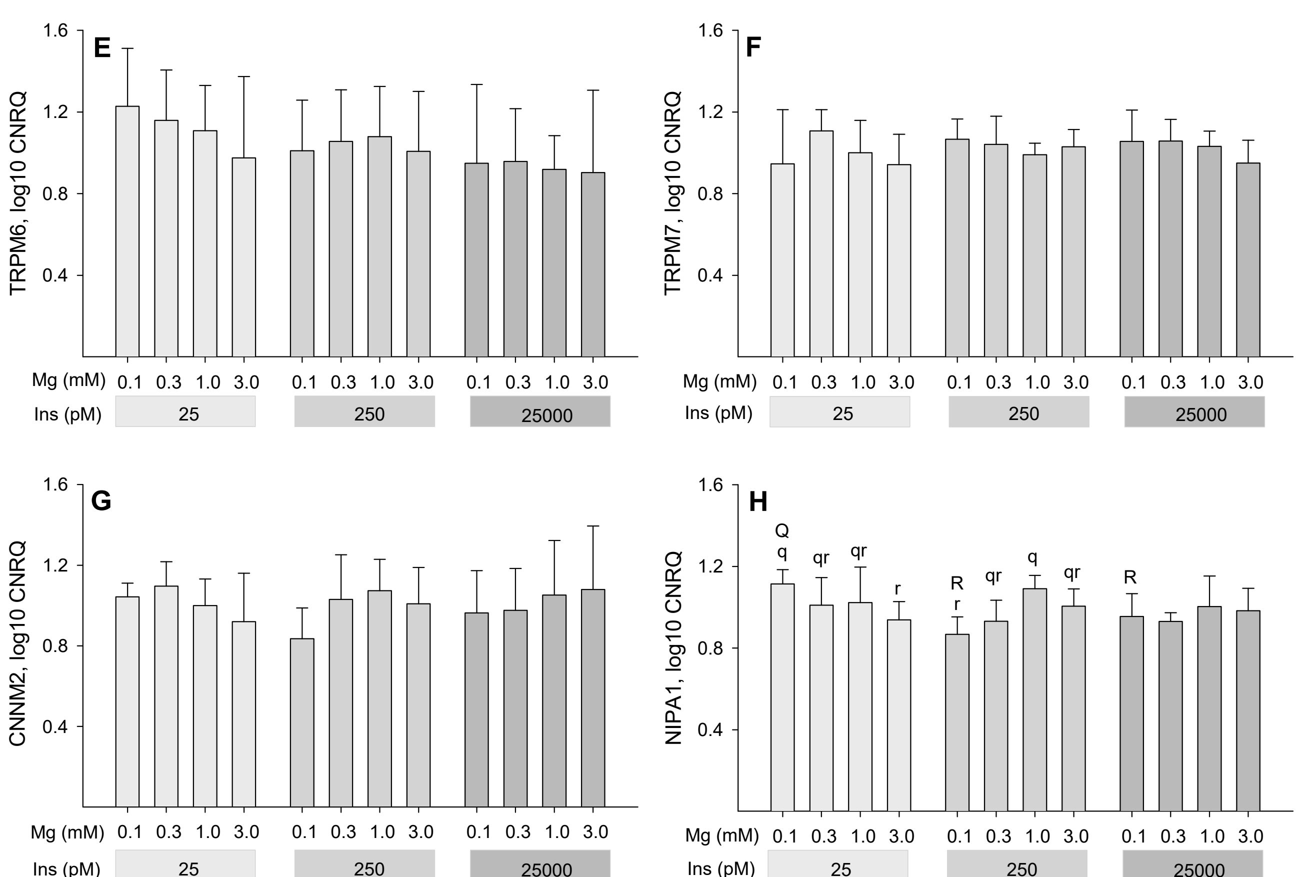


**Figure 4.** Relative mRNA expression (log10 CNRQ) of magnesium-responsive genes *SLC41A1* (**A**), *SLC41A2* (**B**), *SLC41A3* (**C**), *MAGT1* (**D**), *TRPM6* (**E**), *TRPM7* (**F**), *CNNM2* (**G**) and *NIPA1* (**H**) in bovine adipocytes. Cells were cultivated in differentiation media containing various concentrations of insulin (Ins) and magnesium (Mg) for 7 days. Results are given as means from six animals ± SD with three replicates. * Asterisks indicate smaller LSM at 3 mM compared to 0.3 mM Mg concentration ($P \leq 0.05$). Superscript letters a and b indicate different LSM for the factor insulin ($P \leq 0.05$). Lower case letters r and q indicate differences within a given insulin concentration ($P \leq 0.05$) and Capital letters R and Q indicate differences within a given Mg concentration ($P \leq 0.05$) whenever interaction between Mg × insulin was significant.

The expression of the solute carrier family 41 member 1 (*SLC41A1*) was affected by both insulin ($P = 0.021$) and Mg concentration ($P = 0.050$) with no Mg × insulin interaction ($P = 0.72$; Figure 4A). Across Mg concentrations, *SLC41A1* expression was lower at the extremely low insulin concentration of 25 pM compared to the highest insulin concentration of 25,000 pM ($P \leq 0.05$). Within the significant factor Mg, a trend for down-regulation was observed at the lowest Mg concentration (0.1 mM) compared to the highest Mg concentration (3 mM; $P = 0.062$).

No main effects of insulin or Mg were observed for solute carrier family 41 member 3 (*SLC41A3*). However, significant interaction was evident between Mg × insulin ($P = 0.006$; Figure 4C). The interaction identified that log10 CNRQ was lower when 0.1 mM Mg was compared to the other tested Mg concentrations at 250 pM insulin ($P \leq 0.05$ each) and when 250 pM vs. 25 pM insulin concentrations were compared at 0.1 mM Mg concentration ($P \leq 0.05$).

The Mg transporter 1 (*MAGT1*) was significantly affected by the factor insulin ($P = 0.022$) with an up-regulation at 25 pM insulin compared to 250 pM ($P \leq 0.05$; Figure 4D). The effect of Mg concentration was also significant ($P = 0.031$), with a decrease in the LSM of log10 CNRQ when increasing Mg concentration from 0.3 mM to 3 mM ($P \leq 0.05$). Interaction of Mg × insulin was not significant ($P = 0.91$) for *MAGT1* expression.

The main effects of Mg and insulin were not significant for the NIPA Mg transporter 1. However, a significant interaction between Mg × insulin was found ($P = 0.011$; Figure 4H).

At the lowest insulin concentration of 25 pM, log10 CNRQ of *NIPA1* was highest at 0.1 mM and lowest at 3 mM Mg concentration ($P \leq 0.05$). At an insulin concentration of 250 pM, however, log10 CNRQ of *NIPA1* was lowest at 0.1 mM and highest at 1 mM Mg concentration ($P \leq 0.05$). As a consequence, log10 CNRQ of *NIPA1* was higher at 25 pM vs. 250 pM insulin ($P \leq 0.05$) when compared within the 0.1 mM Mg treatments.

**3. Discussion**

Adipose tissue is an important energy reservoir. It plays a crucial role during the transition period when cows run into negative energy balance due to the onset of lactation [21]. Several adipose tissue deposits release their stored energy during this period with prominent contributions from visceral and subcutaneous adipose tissue. These two sources show gradual differences in their expression of anti- and prolipolytic receptors [22] and their proneness to inflammation [23]. However, their responses to changing energy status appear almost identical [22–24].

Insulin is a key signal that reports the energy status to adipocytes. It influences their metabolism, endocrine function and thereby whole-body energy homeostasis [6]. An additional role of Mg in adipocyte function is less well documented, especially in dairy cows. Appreciation of a combined role of Mg and insulin and, in particular, their functional interaction could provide novel prophylactic and therapeutic strategies against metabolic diseases of high-milk-producing cows in the period after calving. Therefore, the present study aimed at obtaining a better understanding of the complex interplay between lipid and carbohydrate metabolism and the role of insulin and Mg in cultured bovine adipocytes. We demonstrated earlier that cells cultured with our protocol express GLUT4, fatty acid binding protein FABP4, fatty acid synthase and peroxisome proliferator activated receptor PPARγ as key markers verifying their adipocyte functionality [25]. Using this model, we tested the effects of different concentrations of insulin and Mg in a two-factorial design on the accumulation of non-polar lipids, glucose uptake capacity, GPDH-activity and on the expression of various Mg-responsive genes in bovine adipocytes.

It is well documented that insulin has effects on adipocytes that are contrary to those of catecholamines. Insulin stimulates glucose uptake, transport of fatty acids into adipocytes, and lipogenesis [26]. Coherent with that general concept, insulin had a positive effect on the accumulation of intracellular non-polar lipids after 14 and 21 days of culture. Stimulation of lipid accumulation was maximized when proceeding from an extremely low insulin concentration of 25 pM to a high physiological insulin concentration of 250 pM and was not stimulated any further by increasing insulin concentration to supraphysiological levels (25,000 pM). This underlines both the functional relevance of our results and the appropriateness of the experimental model that represents adipocytes derived from ruminating cattle. The applied concentration range between 25 and 250 pM insulin largely mirrors the situation in vivo because, unlike humans and most other mammals, blood insulin concentration can be extremely low in postparturient dairy cows in severely negative energy balance. Weber et al. [27] reported that mean insulin concentrations dropped from ~250 pM at 3 weeks prepartum to ~25 pM at 2 weeks postpartum in dairy cows that developed fatty liver syndrome. Thus our in vitro model confirmed that high physiological insulin concentrations have adipogenic and protective functions against metabolic impairment, whereas a low insulin concentration caused lower incorporation of non-polar lipids and may eventually promote apoptosis of adipocytes [28].

The key intention of our study was to demonstrate an additional involvement of Mg in this process because it is known that insulin signaling is dependent on Mg [15]. In support of our hypothesis, extreme Mg starvation with only 0.1 mM Mg in the medium strongly impeded lipid accumulation at all insulin concentrations, at least, after 14 d of culture.

The adipocytes of our study had different substrate sources for lipid synthesis, glucose, acetic acid and serum lipids. It is established textbook knowledge that insulin stimulates the uptake of glucose through the glucose transporter GLUT4 and thereby stimulates fatty acid synthesis [29]. To investigate the influence of Mg on the insulin-dependent

glucose uptake, a 6-NBDG assay was performed. The normalized 6-NBDG uptake in bovine adipocytes partly mirrored that of the lipid/nuclei ratio with a trend to increasing glucose uptake capacity with increasing insulin concentrations at day 14 and a depression of normalized 6-NBDG uptake at extreme Mg starvation (0.1 mM Mg) at day 21. The fact that insulin effects on glucose uptake capacity appeared subtler than insulin effects on lipid accumulation are coherent with the specific characteristics of adipose tissue of dairy cows. It has been shown that GLUT4 protein decreases in bovine adipose tissue already during postnatal development [30] and again with the start lactation [31]. Therefore, the limited insulin-responsiveness of glucose uptake in our study further emphasizes the applicability of our model because it resembles the situation of adult ruminants in vivo where GLUT1 appears to be a dominant glucose uptake pathway [32,33]. Irrespective of the subtle insulin effect, however, a positive effect of Mg on glucose uptake was evident. This effect might be mediated by Mg stimulation of the mammalian TOR complex 2 (mTORC2) [34], leading to GLUT1 phosphorylation and subsequently increased glucose uptake via this transporter [35]. This may imply that the contribution of adequate Mg supply to glucose uptake is not only dependent on the modulation of insulin action as has been proposed in human patients with diabetes mellitus [36,37], but may also include insulin-independent targets, especially in ruminants. Unfortunately, current knowledge about the Mg-insulin-glucose interplay is scarce in dairy cows. The few available studies mostly focused on the role of insulin for Mg status but not vice versa, the role of Mg for insulin effects [38,39].

Our third functional assay investigated insulin and Mg effects on the activity of GPDH, i.e., the enzyme that catalyzes reversible conversion of dihydroxyacetone phosphate and NADH into glycerol-3-phosphate and $NAD^+$. The enzyme provides glycerol from carbohydrate metabolism to triglyceride synthesis [40]. Previously, it was shown that GPDH activity of bovine adipocytes is dependent on the presence of BSL, PPARγ agonist, dexamethasone and insulin [41]. In that previous study, complete omission of insulin from the culture medium reduced GPDH activity by 68% compared to the presence of 280 nM insulin [41]. That setup with complete omission of insulin was noticeably different from our setup where three levels of insulin treatment were compared. In our study, GPDH activity was influenced by both insulin and Mg with highest enzyme activities being observed at the lowest insulin concentration and the highest Mg concentration. Although the mechanisms behind that are not fully clear at present, this finding has important functional implications. Postparturient dairy cows regularly suffer from increased blood concentrations of non-esterified fatty acids that may cause fatty liver, ketosis and associated diseases [6]. Our present results suggest that Mg availability above physiological requirement may promote the synthesis of glycerol in adipocytes in the face of low plasma glucose and low plasma insulin concentrations and thus help sequestrating excessive serum lipids. Such scenario would help to ameliorate lipid accumulation in other tissues, e.g., fatty liver.

A final intention of the present study was to investigate the response of a set of so-called Mg-responsive genes to varying insulin and Mg concentrations. The Mg-responsiveness of many of these genes is known from previous experiments applying either dietary Mg restriction in mice or applying different Mg concentrations in cell culture experiments. For a comprehensive review see Kolisek et al. [42].

The expression of *SLC41A2*, *TRPM6*, *TRPM7* and *CNNM2* was not significantly affected by the availability of Mg and insulin in the present study. For the epithelial Mg channel TRPM6 [43] and the Mg-homeostatic factor CNNM2 [44], this fact may simply relate to the very low and variable expression observed in bovine adipocytes. For *TRPM7* this was not unexpected since the expression of *TRPM7* as a main entry mechanism for $Mg^{2+}$ into cells seems to be constitutive [45]. Specifically for adipocytes (3T3-L1 cells), however, it was shown that TRPM7 channels contribute to adipogenesis and their deactivation impairs differentiation [46].

SLC41A1 as a $Na^+$-dependent Mg exchanger is ubiquitously expressed [18] and its transport activity is stimulated by phosphorylation via cAMP-dependent protein kinase A (PKA) [47]. The latter results in higher Mg efflux and therefore a decrease in intracellular

Mg concentration. Insulin signaling inherently counteracts PKA-dependent SLC41A1 activation and thus supports intracellular Mg retention [48]. In the present study, the mRNA expression of *SLC41A1* increased with increasing insulin concentrations and tended to increase with increasing Mg concentrations. A lower expression of SLC41A1 at low Mg concentration makes perfect sense because this would help to sequester Mg inside the cells when extracellular Mg availability is limiting. On the other hand, the inhibitory action of insulin on SLC41A1 function will also increase intracellular Mg levels [48], thus explaining a compensatory up-regulation of *SLC41A1* gene expression by high insulin concentrations to avoid excessive intracellular Mg accumulation, especially at concurrently high external Mg availability.

The transporter SLC41A3 has recently been characterized as a $Na^+$-dependent $Mg^{2+}$ extruder of the inner mitochondrial membrane [49]. In our experiments, the expression pattern of *SLC41A3* at 250 pM insulin mirrored the Mg effect on *SLC41A1*; therefore, equally suggesting that a downregulation of this transporter may rescue mitochondrial Mg concentration at low Mg availability. However, based on significant statistical interaction, this was obvious only as long cytosolic Mg concentration was protected by a high physiological insulin concentration of 250 pM. At 25 pM insulin, Mg deficiency combined with an insufficient insulin concentration seemed to stimulate the expression of the gene. If translated into functional SLC41A3, this might result in stronger extrusion of $Mg^{2+}$ from mitochondria to sustain Mg availability in the cytosol [49,50].

The mRNA expression of *MAGT1* was up-regulated at lower Mg (0.3 mM vs. 3 mM) and lower insulin concentrations (25 vs. 250 pM) in the present study. The regulation of *MAGT1* expression was thus almost inverse to that of *SLC41A1*. This would be compatible with inverse regulation of and *SLC41A1* as a Mg efflux system and *MAGT1* as a Mg influx system, provided the latter postulate is correct [51,52].

An accepted electrogenic Mg influx pathway is NIPA Mg Transporter 1 (NIPA1). Previous studies showed that the *NIPA1* expression is dependent on the extracellular Mg concentration, with increased expression at low Mg concentrations [53,54]. In the present study, we identified the presence of *NIPA1* in bovine adipocytes with a significant Mg $\times$ insulin interaction. We detected up-regulation of *NIPA1* upon an undersupply (0.1 mM) of Mg in comparison to an oversupply (3 mM) only at the very low insulin concentration of 25 pM, confirming the results of Goytain et al. [53]. However, this pattern was inverted or neutralized at higher insulin concentrations, possibly indicating attempts to increase intracellular Mg retention upon increasing insulin availability as outlined in the discussion on SLC41A1 in a previous paragraph.

In conclusion, the present study investigated the effects of Mg and insulin on the differentiation of cultured bovine preadipocytes to mature adipocytes and lipogenesis. We demonstrated that insulin and Mg jointly promote these processes. An influence of insulin on glucose uptake capacity was only seen as a trend, which underlines the functional dominance of insulin-independent glucose uptake pathways that are inherent to our culture model and relevant for adipose tissue of adult cows. Mg influenced glucose uptake capacity of mature adipocytes positively and a supraphysiological Mg concentration of 3 mM increased GPDH activity, specifically at concurrently low insulin concentrations. The latter findings have the important pathophysiological implication that oversupply of Mg may have a potential to rescue the production of glycerol for triglyceride synthesis even in the case of low blood glucose and insulin concentrations. This could promote re-esterification of excessively circulating non-esterified fatty acids in adipocytes and thus counteract lipid injury to other organs, i.e., fatty liver and ketosis. The analysis of Mg-responsive genes further supported the important role of Mg in adipocytes during negative energy balance by demonstrating that certain genes that may rescue cytosolic Mg concentration during Mg starvation (*NIPA1* and *SLC41A3*) are specifically up-regulated at low insulin concentrations.

## 4. Materials and Methods

Bovine serum lipids (BSL) (Ex-Cyte), fetal bovine serum (FBS), penicillin-streptomycin, acetic acid, cell culture medium DMEM and Dulbecco´s phosphate-buffered saline (DPBS) were purchased from Merck Millipore (Darmstadt, Germany). N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), ascorbic acid, amphotericin B, biotin, bovine insulin, Nile red, trypan blue and D-glucose were obtained from Sigma Aldrich (Taufkirchen, Germany). 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Roche (Grenzach-Wyhlen, Germany). Magnesium chloride solution was obtained from Honeywell Fluka™ (Taufkirchen, Germany). The 6-NBDG (6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose) was purchased from Life Technologies (Darmstadt, Germany). The Glycerol-3-Phosphate Dehydrogenase (GPDH) Activity Colorimetric Assay Kit was acquired from BioVision Inc. (Milpitas, CA, USA).

The NucleoSpin® RNA kit was purchased from Macherey-Nagel GmbH & Co. (Düren, Germany). The iScript™ cDNA Synthesis Kit was obtained from Bio-Rad Laboratories GmbH (Munich, Germany). All primers and probes were synthesized by Eurofins Genomics (Ebersberg, Germany). Cell culture flasks were from Techno Plastic Products (Trasadingen, Switzerland), 24-well cell culture plates (CytoOne) were from Starlab (Hamburg, Germany), 96-well plates were sourced from Carl Roth (Karlsruhe, Germany) and 384-well plates were from Biozym Scientific GmbH (Hessisch Oldendorf, Germany).

### *4.1. Adipose Tissue Collection*

Collection of adipose tissue was in accordance with the German legislation on animal welfare. All experiments were carried out using tissues from animals slaughtered for human consumption; i.e., no animals were specifically raised and killed to perform these experiments. Therefore, no animal use and care approval was required. Bovine subcutaneous adipose tissue was aseptically collected from the neck region (at the level of the 2nd to 3rd cervical vertebra) of exsanguinated Holstein cattle (median age, 9.5 months). Isolating stem cells from ~5 to 12 month-old cattle has the great advantage that these young animals have a larger pool of mesenchymal stem cells than older animals. On the other hand, cattle of this age are fully ruminating and thus represent the situation of lactating cows rather well. Explant culture and passage of pre-adipocytes was performed as described by Jurek et al. [25].

### *4.2. Induction and Differentiation of Adipocytes*

For the evaluation of non-polar lipids and the measurement of glucose uptake, $1.5 \times 10^4$ cells/mL were transferred into 24 well culture plates. For GPDH assay and RT-qPCR experiments, cells were seeded into T-75 flasks at a confluency of 30% and grown until 100% confluency. Cell passaging was implemented as described by Jurek et al. [25].

After reaching confluence, differentiation of pre-adipocytes into adipocytes was induced for 2 days as published by Jurek et al. [25]. For the subsequent experiments, differentiated adipocytes were kept in 12 different types of adipocyte differentiation media. Differentiation media were based on DMEM (without glucose, without Mg, with 4 mM stable L-glutamine and 15 mM HEPES) supplemented with D-glucose (10 mM), penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), amphotericin B (2.5 µg/mL), biotin (10 µM), ascorbic acid (113 µM), acetic acid (20 mM) and bovine serum lipids (5 µL/mL). This basic medium was complemented with 25 pM, 250 pM or 25,000 pM bovine insulin together with 0.1 mM, 0.3 mM, 1 mM or 3 mM magnesium ($Mg^{2+}$) in a two-factorial design. Depending on the experiment and described below, cells were kept for 7, 14 or 21 days in the differentiation medium. The incubation was performed in a humidified atmosphere at 37 °C with 95% air and 5% $CO_2$. The medium was replaced after every 48 h with 1 mL/well or 10 mL/T-75 flask.

### *4.3. General Procedures*

#### 4.3.1. Measurement of Non-Polar Lipids

The content of intracellular, non-polar lipids was measured after 14 and 21 days in differentiation medium in 24-well plates. The protocol for staining, imaging and evaluation has been described by Sandhu et al. [55]. The fluorescence signal of Nile red (lipid index) was divided by the fluorescence signal of DAPI (nuclei index) to obtain the lipid/nuclei ratio, which represents the concentration of non-polar lipids corrected for cell density.

#### 4.3.2. Measurement of 6-NBDG Uptake

Uptake of 6-NBDG as surrogate for the uptake capacity for glucose was assessed after 14 and 21 days of cultivation in the above described differentiation media. 24 h before measurement, adipocytes were washed two times for 5 min with warm DPBS (without magnesium, without calcium). Subsequently, 1 mL/well DMEM (without glucose, with 4 mM L-glutamine), supplemented with D-glucose (5.5 mM), penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), amphotericin B (2.5 µg/mL) and HEPES (15 mM) was added. After 24 h, cells were washed two times for 5 min with warm DPBS (without magnesium, without calcium) in order to incubate the cells with 0.4 mL/well DMEM (without glucose, with 4 mM L-glutamine) supplemented with penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), amphotericin B (2.5 µg/ mL), HEPES (15 mM) and various insulin concentrations (25 pM, 250 pM or 25,000 pM) for 1 h. After removing the medium, cells were washed again two times for 5 min with warm DPBS (without magnesium, without calcium). DPBS was discarded and the cells were incubated with 0.4 mL/well of 6-NBDG (150 µM solved in glucose-free DMEM) at 37 °C for 30 min. After incubation, 6-NBDG was removed and cells were washed three times with ice cold DPBS (without magnesium, without calcium) with 0.5 mL/well DPBS remaining after the last washing step. The uptake of 6-NBDG was measured at 485/530 nm (excitation/emission; ex./em.) in a Multimode Plate Reader (PerkinElmer, MA, USA) in 24-well plates at 37 °C. Afterwards, DPBS was removed and the cells were incubated for 5 min with 0.5 mL/well DAPI (0.2 µg/mL in DPBS). DAPI fluorescence was measured at 358/461 nm (ex./em.) in the same Multimode Plate Reader.

The fluorescence signal of 6-NBDG (glucose index) was divided by the fluorescence signal of DAPI (nuclei index) to obtain the glucose/nuclei ratio, which represents the glucose uptake capacity corrected for variation in cell density.

#### 4.3.3. Glycerol 3-Phosphate Dehydrogenase (GPDH) Assay

Cultured adipocytes (7 days) were washed two times with warm DPBS (without magnesium, without calcium) and trypsinized in the same way as for passaging [25]. For stopping the trypsin reaction, cells were resuspended in DMEM (with 10% FBS and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively) and centrifuged at 350× *g* for 5 min at room temperature. The cell pellet was resuspended in 5 mL DPBS (without magnesium, without calcium). Subsequently, cells were stained with trypan blue and counted manually in a Neubauer cytometer. The measurement was conducted according to the manufacturer´s instruction with $1 \times 10^6$ cells as duplicate in 96-well plates with an NADH standard curve and positive controls. The absorbance was measured at 450 nm in a Multimode Plate Reader (Tristar LB942, Berthold Technologies, Bad Wildbad, Germany) at 37 °C for 1 h.

#### 4.3.4. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) of Magnesium-Responsive Genes

For RNA isolation, differentiated adipocytes were collected after 7 d as described by Jurek et al. [25] and immediately processed. RNA-isolation was performed by means of NucleoSpin® RNA kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer´s instructions. The integrity, purity and quantity of RNA was assessed by using the Bioanalyzer RNA 6000 Nano assay (Agilent Technologies, Santa Clara, CA,

USA). All samples used had an RNA integrity number (RIN) ≥ 8. Aliquots of 1000 ng RNA of each sample were reverse transcribed to cDNA by using the iScript™ cDNA Sythesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany) according to the manufacturer´s instructions in a Mastercycler™ Nexus Gradient (Eppendorf GmbH, Hamburg, Germany) in a one-step protocol (one cycle: priming at 25 °C for 5 min, reverse transcription at 46 °C for 20 min and inactivation of the transcriptase at 95 °C for 1 min).

Primers and probes for RT-qPCR are listed in Table 2. Three reference genes were used for normalization (β-actin [*ACTB*], ribosomal protein S19 [*RPS19*] and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta [*YWHAZ*]). RT-qPCR was carried out in a Viia 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with Biozym Probe qPCR Kit separate ROX (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). The program consisted of initial denaturation at 95 °C for 2 min, and 40 cycles with denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 20 s.

**Table 2.** Primer and probe sequences, applied concentrations (nM), as well as expected amplicon sizes (bp) and database accession numbers of magnesium-responsive genes and reference genes.

| Gene | Sense Primer (5′-3′) / Anti-Sense Primer (3′-5′) / Probe | nM | Ampl. Size (bp) | Database Accession No. |
|---|---|---|---|---|
| ***SLC41A1*** | **TGGTGTTCCTCTATACCATCAG** | **1000** | 186 | NM_001206036.1 |
| | TCAAGTACGGGATGGAGAAG | 3000 | | |
| | ATGTAGAGCAGGATCAGCACCTGGAGCAGA | 500 | | |
| ***SLC41A2*** | CTGCTTTTAGTGATACCTGGAC | 500 | 178 | NM_001205910.1 |
| | TTCCTTTCCTCCAGAAATGATG | 1500 | | |
| | TTGCTGTGGATCGCTGACTGGATG | 150 | | |
| ***SLC41A3*** | CTTCTGCACTATTTCCAGCAC | 1500 | 100 | XM_024983333.1 |
| | TCATCTCCAGGTTGCCCTTC | 3000 | | |
| | TTCACGGAGATGAAGGACCTGCTGACCTTGG | 500 | | |
| ***MAGT1*** | GCTCAATTTGTAGCTGAAACAC | 500 | 124 | NM_001244318.2 |
| | CACACATTATCTTTCGCTTTCC | 1500 | | |
| | ATGTGAAGCTGCTACATCTGACATGGATATTG | 150 | | |
| ***CNNM2*** | GCTCCAGAATACTACCTCTACC | 500 | 83 | NM_001191172.1 |
| | GCTTCTACTTCACTTTCCCC | 1500 | | |
| | CGAAACAAACCTGTAGACTACTTCGTTCTCAT | 150 | | |
| ***NIPA1*** | TCCCCGAAATCTGAGAGTGTG | 1000 | 115 | XM_002685192.6 |
| | AGAAGATGAGCAGCAGCAGC | 1500 | | |
| | TGGAGGAGAAGCTGACCAATCCAGTGTTTGTG | 150 | | |
| ***TRPM6*** | ACAAACCATTCCCTACACTCC | 500 | 125 | XM_015472505.2 |
| | CGTTGTTGTTGTTGTACTTCC | 1500 | | |
| | TTGACCATCGAGAAGTATATGACGGGGGAG | 150 | | |
| ***TRPM7*** | ATACAAGAGGGGAGTTACTGG | 500 | 112 | NM_001206166.3 |
| | GGGCCAAAAACCATATCACAG | 1500 | | |
| | CTGACCCATCTGTGATAAAGGCAGAAGAA | 150 | | |
| ***RPS19*** | GGAAAAGGACCAAGATGGGG | 500 | 136 | NM_001037467.2 |
| | CGAACGAGGCAATTTATTAACC | 1500 | | |
| | ACAGAGAGATCTGGACAGAATCGCTGGACA | 150 | | |
| ***YWHAZ*** | AGAGAGAAAATAGAGACCGAGC | 500 | 144 | NM_174814.2 |
| | AGCCAAGTAGCGGTAGTAG | 1500 | | |
| | CCAACGCTTCACAAGCAGAGAGCAAA | 150 | | |
| ***ACTB*** | GCCAACCGTGAGAAGATGAC | 500 | 124 | NM_173979.3 |
| | AGTCCATCACGATGCCAGTG | 1500 | | |
| | CCAGATCATGTTTGAGACC TTCAACACCCCTGC | 150 | | |

Reactions were performed in final volumes of 9.9 μL, containing 4.5 μL of cDNA, 4.5 μL Biozym mastermix, 0.3 μL of each sense and antisense primers and probes as indicated in Table 2. All reactions were performed in triplicate. An inter-run calibrator (IRC) was utilized to correct for variations between the different runs and a no-template-control (NTC) was included for monitoring contamination and primer-dimer formations.

Thresholds were automatically calculated by the Viia 7 software. For data analysis, the software qbasePLUS (Biogazelle NV, Zwijnaarde, Belgium) was used to perform inter-run calibration, determining dilution series-based gene specific amplification efficiencies and testing for expression stability of reference genes. After normalization of Cq values with the respective reference gene(s), results were exported as calibrated normalized relative quantity (CNRQ) values which were used for statistical analysis.

### *4.4. Statistical Analysis*

All presented data were statistically analyzed and all graphs plotted by using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). Experiments to determine lipid/nuclei ratio and glucose/nuclei ratio were conducted in duplicates with cells from six animals. Analysis of GPDH activity was conducted in duplicates with cells from five animals. All multiple measurements were arithmetically pooled per animal and, if applicable, adjusted by subtracting blank values and matched to standard curves. All experiments were analyzed by two-way repeated measures analysis of variance (ANOVA) for the factors "animal", "insulin" (25 pM, 250pM and 25,000 pM) and "Mg" (0.1 mM, 0.3 mM, 1 mM and 3 mM). Furthermore, possible interactions between Mg × insulin were tested.

The RT-qPCR data were retrieved from cells of six animals in triplicates, where each CNRQ value originated from triplicate RT-qPCR analysis. The RT-qPCR data sets were analyzed by two-way analysis of variance (ANOVA) with the effects of "insulin" (25 pM, 250pM and 25,000 pM), "Mg" (0.1 mM, 0.3 mM, 1 mM and 3 mM) and their two-way interaction.

If overall analysis of data showed statistical significance ($P \leq 0.05$), differences between groups were identified by the Holm-Sidak post-hoc test. If normality test failed, data were log-transformed before statistical evaluation.

Data are presented as means ± standard deviation (SD) for individual groups or least square means (LSM) ± pooled standard error of means (SEM) for main effect data. Statistical significance was considered at $P \leq 0.05$; trends are mentioned if $0.05 < P \leq 0.1$. The number of studied animals is given as $n$.

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## Abbreviations

| | |
|---|---|
| 6-NBDG | 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose |
| ACTB | β-actin |
| Akt (PKB) | protein kinase B |
| ANOVA | analysis of variance |
| BSL | bovine serum lipids |
| cAMP | cyclic adenosine monophosphate |
| cDNA | complementary DNA |
| CNNM2 | cyclin and CBS domain divalent metal cation transport mediator 2 |
| CNRQ | calibrated normalized relative quantity |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DPBS | Dulbecco's phosphate-buffered saline |
| em. | emission |
| ex. | excitation |
| FBS | fetal bovine serum |
| GLUT | glucose transporter |
| GPDH | glycerol 3-phosphate dehydrogenase |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| Ins | insulin |
| IRC | inter-run calibrator |
| IRS | insulin receptor substrate |
| LSM | least square means |
| MAGT1 | magnesium transporter 1 |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| NADH | reduced nicotinamide adenine dinucleotide |
| NIPA1 | Nipa magnesium transporter 1 |
| NTC | no template control |
| PDE3b | phosphodiesterase 3b |
| PDK1 | phosphoinositide-dependent kinase-1 |
| PI3K | phosphoinositide 3-kinase |
| PKA | protein kinase A |
| RIN | RNA integrity number |
| RNA | ribonucleic acid |
| ROX | carboxy-X-rhodamine |
| RPS19 | ribosomal protein S19 |
| RT-qPCR | reverse-transcription quantitative polymerase chain reaction |
| SD | standard deviation |
| SEM | standard error of mean |
| SLC | solute carrier family |
| STT3B | STT3 oligosaccharyltransferase complex catalytic subunit B |
| TRPM | transient receptor potential cation channel subfamily M |
| YWHAZ | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta |

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# Chapter 7: Discussion

Research into insulin imbalances and insulin resistance has been steadily increasing over the last few years. Diseases affecting insulin action are on the rise in humans, and so the study of, for example, diabetes mellitus including secondary diseases is becoming more and more relevant (Fox et al. 2006; Ginter and Simko 2012; Olokoba et al. 2012). These emerging and increasing modern diseases also affect dairy cows (De Koster and Opsomer 2012) because of the intensive breeding for higher milk yields (Berglund 2008) and also the provision of modified rations to achieve this performance. A similar disease pattern, namely insulin resistance (De Koster and Opsomer 2013), has been known for several years to be manifested in high-yielding cows during the transition period (Chalmeh et al. 2015). The consequences are diseases such as ketosis, fatty liver, milk fever, and inflammatory diseases and therefore a decrease in the performance and life time of cows (Herdt 2000; Mulligan and Doherty 2008; Sordillo and Raphael 2013). The associated health consequences place a financial burden on farmers, and this, in turn, creates an ethical problem because of unprofitability and consequently culling (Oltenacu et al. 1984; Miller and Dorn 1990; Kossaibati and Esslemont 1997; Croney and Anthony 2011). Thus, research into the causes of these diseases and possible treatment approaches are attracting special interest (Drackley 1999; Fleischer et al. 2001). High-performance cattle and their problems are moving into the spotlight, as can be seen from the rising numbers of publications based on this problem. An understanding of the mechanisms of insulin resistance in dairy cows is of fundamental importance, and detailed investigations into its interconnections, explicitly those to adipose tissue, are vital (De Koster et al. 2016; Contreras et al. 2017b).

## Cultivation of adipose derived stem cells (ASC)

The ease of availability and the many possibilities that ASC offer has increased research interest in these cells. In human medicine, ASC have been shown to be versatile, for example, in the treatment of tissue defects or in the treatment of autoimmune diseases (Lindroos et al. 2011). Their advantage lies in that the cells can be easily harvested, cultured, and differentiated. If autologous cells are used, no rejection reactions occur because the donor and recipient are the same person (Gaiba et al. 2012). Furthermore, their popularity has increased because they are self-renewing and multipotent, which means that they can differentiate into various cell types in vitro, such as adipocytes, chondrocytes, osteocytes, or even endothelial cells, thereby facilitating research into these cells (Strem et al. 2005). The advantages and thus the research interest in ASC have also been noted in veterinary medicine (Marx et al. 2015). The aim of this thesis has therefore been to develop a reliable and robust method to obtain, culture, and differentiate (**chapter 4 and 5**) bovine ASC into adult adipocytes. These

cells represent a valuable *in vitro* model for studying the influence of magnesium and insulin on adipocytes in order to improve our understanding of cellular processes and to allow conclusions to be drawn with regard to possible diseases during the transition period (**chapter 6**).

Progenitors of adipocytes, namely preadipocytes, can be readily cultivated from various sources of white adipose tissue (WAT), for example, from subcutaneous or visceral fat (Cignarelli et al. 2016), muscle fat (Albrecht et al. 2015), or bone marrow fat depots (Jeong et al. 2015). A number of protocols are currently available, many of them focusing on the digestion of adipose tissue (Bakker et al. 2004; Carswell et al. 2012; Storck et al. 2015). In the preliminary work for this research project, we investigated the best source and cultivation method for preadipocytes (data not shown). These experiments showed that younger animals have a larger and more active pool of stem cells in adipose tissue than older individuals, a finding that is reflected in many research results (Karagiannides et al. 2001; Sepe et al. 2011) and that we were able to confirm. We tried to find the best source of preadipocytes by taking the adipose tissue from subcutaneous regions of several body sites, such as the umbilical, flank, or neck region, the adipose tissue from the intra-abdominal region, such as the greater omentum, or the adipose tissue entrapping organs. Various cultivation methods and protocols were tested with these tissue samples. These included the above-mentioned digestion method and methods of ceiling (Fernyhough et al. 2004) and explant (Jing et al. 2011) cultivation. Although many publications suggest that sampling from the greater omentum and the digestion method bring success, our experiments have shown that the subcutaneous depots in the neck region (at the level of $2^{nd}$ to $3^{rd}$ cervical vertebra) of calves younger than 11 months, cultured as explants, are the most productive with regard to cell number and cell type and to the supply of the purest cell cultures of bovine ASC. With a view to further possible clinical trials, the removal of subcutaneous fat tissue as a biopsy and the cultivation of this sample as an explant represented the easiest and most cost-effective way to perform trials on ASC. Of course, differences, especially in the expression of receptors of the lipolytic pathways (Weber et al. 2016b) or in the tendency to inflammatory processes (Ji et al. 2014), can be seen depending on the origin, e.g., subcutaneous or visceral, of the adipose tissue samples. However, the reaction attributable to the variable energy status is almost the same between subcutaneous and visceral fat in cattle (Weber et al. 2016b; Weber et al. 2016c) unlike in humans. Our first aim was the provision of evidence that the cells were definitely ASC or committed preadipocytes. For this purpose, we allowed the cells to grow out of the explant into the so-called base medium (**chapter 4 and 5**), and as soon as they grew out, we removed the explant. Once the cells had reached confluency, the medium was switched to the preadipocyte medium. These media differed in their fetal bovine serum (FBS) content and were based on

formulations from Riedel (Riedel 2013) and Hendijani (Hendijani 2017). FBS provides a wide variety of macromolecules, proteins, lipids, trace elements, and other nutrients, plus hormones and growth factors, making it an integral part of cell culture (Gstraunthaler 2003). The outgrowth capacity of the putative preadipocytes in these media was tested, and the identity of these cells was assessed. To confirm that these cells were indeed mesenchymal stem cells, several criteria were investigated. These included their attachment to plastic, their expression of a certain cluster of differentiation markers (CD markers), and their multipotency (Dominici et al. 2006; Brown et al. 2019). Our experiments showed that the cells attached very well to plastic, and that they were multipotent. With the help of osteogenic differentiation medium, we were able to demonstrate that the ASC were also able to differentiate into osteocytes and to accumulate minerals (**chapter 5**). Multipotency was thus an established feature of these cells. Furthermore, ASC should express the markers CD73 (NT5E; ecto-5'-nucleotidase), CD90 (Thy-1 cell surface antigen), and CD105 (ENG; endoglin) as a minimum requirement (Schaffler and Buchler 2007). Although ASC are known to express the CD markers, changes in their expression pattern depending on their degree of differentiation and on the various medium supplements have not yet been fully investigated in adipocytes. In **chapter 4 and 5**, we confirmed gene expression by qPCR and protein expression by immunofluorescence staining with antibodies directed against these surface markers. Our preadipocytes were positive for these markers, whereas their expression decreased as soon as the preadipocytes underwent differentiation into adipocytes, with a concomitant increasing expression of adipocyte markers. Furthermore, we were able to reveal that the expression of these stem cell markers was strongly dependent on FBS (**chapter 4 and 5**). As soon as FBS was present in the differentiation medium, expression increased. This effect was reduced as soon as bovine serum lipids (BSL), a cholesterol-rich substrate, was included and was almost completely absent as soon as FBS was omitted from the medium. Thus, FBS is essential for the cultivation of ASC and, because of its composition, promotes cell replication and the expression of ASC stem cell markers, whereas differentiation is not supported. This changed when BSL was added as this signal increased the differentiation of ASC into adipocytes. In particular, the expression of CD73 and its localization in the cell was strongly dependent on the FBS concentration (**chapter 4**). We were able to demonstrate that FBS causes the movement of CD73 towards the cell surface. CD73 is suggested to play an important role in cell division (Li et al. 2013), a suggestion that fits our observation of FBS increasing the replication of ASC (**chapter 4 and 5**). Overall, our cultivation method with subcutaneous harvesting of ASC and the culture of outgrowing cells from the explants can be summarized as being successful. This was confirmed by the expression of stem cell markers by the cultured cells, whereby FBS had a positive effect on the growth of ASC and the absence of FBS, in conjunction with BSL, increased differentiation into adipocytes. Our protocol thus allowed the cultivation of a large

quantity of multipotent mesenchymal stem cells within a short period of time and with little effort. These cells can be differentiated into various cell types with the aid of an induction and differentiation protocol, e.g., in our case, into adult adipocytes and, as in **chapter 5**, into osteocytes. To induce the expansion of the undifferentiated ASC in order to obtain sufficient numbers of cells, an induction cocktail is required that contains insulin, a glucocorticoid, and a substance that increases the concentration of cAMP (Ntambi and Young-Cheul 2000). The previously published concentrations of these substances, namely insulin, dexamethasone, rosiglitazone, IBMX, and biotin, were high and thus we wished to test to what extent these concentrations could be reduced without weakening the effect of induction. Dexamethasone increases the expression of PPARγ and other nuclear factors (Wu et al. 1996), rosiglitazone activates PPARγ (Spiegelman 1998), and insulin is known for its adipogenic effect which is also based on PPARγ interaction (Caprio et al. 2007). The increase in the concentration of cAMP, which is necessary for induction (Petersen et al. 2008), can be achieved by IBMX so that all the above mentioned reagents are essential for the initiation of the signaling events leading to induction and differentiation. We used the quantities and concentrations of the various components of the induction media given in the literature as a guide and defined them as 100% medium (Riedel 2013; Pu and Veiga-Lopez 2017). The aim was then to reduce the respective substances to 30% and 10% and to investigate whether successful induction could still be achieved or is even improved (**chapter 5**). A reduction of the supplements to 30% induced induction to the same extent as in those cells exposed to 100%. A numerical, but not a statistically significant improvement was achieved by this lower concentration (**chapter 5**). We had thus created an induction cocktail that enabled successful cell induction but with economic concentrations of the respective substances. This protocol was therefore used in all further experiments (**chapter 5 and 6**). The composition of our medium also reduced the risk of exposing the cells to unnecessarily high concentrations of these supplements, which might be detrimental for the cells, especially in the case of dexamethasone (Caprio et al. 2007). After successful induction, a medium had to be created that provided ideal conditions to allow adipocytes to differentiate and grow, while containing sufficient substrate to allow the formation of intracellular lipids. BSL, a substrate derived from bovine serum, were previously shown to increase adipocyte differentiation and lipogenesis (Grant et al. 2008; Beloor et al. 2010). We therefore incorporated BSL into our formulations and examined to what extent it influenced the formation of intracellular lipids and thus lipogenesis during adipogenesis (**chapter 4 and 5**). A differentiation medium with BSL but without FBS was shown to be most effective to promote the accumulation of intracellular fat as confirmed by Nile red staining and quantification of lipids per cell. As soon as FBS was added, even if BSL was present, lipogenesis was reduced compared with BSL alone. Thus, BSL can be regarded as the driving force of adipogenesis and lipogenesis (**chapter 4 and 5**). FBS, on the other hand, increased cell replication and is

thus relevant for cell proliferation, but not for differentiation (**chapter 4**). We were also able to show this effect at the gene expression level and by the immunofluorescence staining of certain proteins, as described above and based on the results of **chapter 4**. Specifically, we investigated the behavior of the expression and synthesis of fatty acid binding protein 4 (FABP4) (**chapter 4 and 5**). *FABP4* is mainly expressed in adipocytes and is regulated by insulin and FA (Floresta et al. 2017). The role of FABP4 is to stimulate lipolysis and inhibit lipogenesis (Zeng et al. 2020) by upregulation of the phosphorylation of HSL (Dou et al. 2020) and is crucial for the transportation of FA and their oxidation (Gan et al. 2015). Furthermore, FABP4 negatively influences PPARγ activity by causing the ubiquitination of PPARγ, which is directly related to the insulin sensitivity of adipocytes (Garin-Shkolnik et al. 2014) by modulating gene expression, phosphorylating molecules of the insulin signal pathway, or both (Kvandová et al. 2016). Thus, FABP4 can be regarded as a marker for the stage of differentiation (Shan et al. 2013; Wojciechowicz et al. 2013) and also serves as a marker for adult adipocytes (Spiegelman et al. 1983; Shan et al. 2013; Furuhashi et al. 2014). The adipocytes that we cultured showed successful gene and protein expression of FABP4, confirming their successful cultivation with our protocol (**chapter 4 and 5**). Various expression patterns could be shown, depending on differentiation, time point, and medium supplements. In **chapter 5**, the supplementation of the medium with BSL but without FBS achieved the highest expression of *FABP4*, namely 10 times higher than that in the media with FBS or with FBS and BSL. This can be explained by the fact that BSL increases adipocyte differentiation by activating PPARγ and thereby stimulating the expression of C/EBP alpha (Grant et al. 2008; Beloor et al. 2010). Subsequently, genes are activated that enable the uptake, transport, and synthesis of long-chain FA (Beloor et al. 2010). We have also confirmed this observation visually, as in the cells cultivated in medium with BSL but without FBS, the immunofluorescence for FABP4 was highest, matching the microscopic images of the stained fat vacuoles of the cells in the same medium (**chapter 4**). Once again, the cells were clearly mature adipocytes. In contrast, this pattern was much reduced in media with FBS, as was also confirmed by the analysis of lipoprotein lipase (LPL) and fatty acids synthase (FAS) in PCR or immunofluorescence studies. Being relevant enzymes of FA metabolism and therefore lipogenesis during adipogenesis, these proteins behaved similarly to FABP4 (**chapter 5**). Interestingly, the microscopic examinations, i.e., the immunofluorescence and the Nile red staining of the fat vacuoles, revealed that the accumulation of fats and the expression of FABP4 took place in clusters (**chapter 4**). The cell layer contained islets in which the cells were more differentiated than in other areas of the cell layer. This indicates that some type of communication occurs between neighboring cells, or even that information and signals are transmitted between the cells. Such signals might be paracrine, for example through leptin or

adiponectin, which play an essential role in energy metabolism such as glucose and fat metabolism (Auwerx and Staels 1998; Kadowaki et al. 2006; Mielenz et al. 2013).

In **chapter 5**, we further optimized the adipogenic transdifferentiation of bovine ASC and investigated the effect of ascorbic acid as a possible promoter of adipogenesis in addition to BSL. Ascorbic acid is known to promote the differentiation of mesenchymal stem cells and has been established in some research groups as a media additive for the differentiation of osteogenic and chondrogenic lineages (Alcain and Buron 1994; Duarte and Lunec 2005). Reasons for this might be that ascorbic acid is important for collagen synthesis, which is relevant for adipogenesis (Weiser et al. 2009; Cuaranta-Monroy et al. 2014), and that ascorbic acid acts as an antioxidant which reduces tissue and DNA damage by ROS during oxidative stress (Sriram et al. 2019). In our experiments (**chapter 5**), ascorbic acid was shown to have a positive effect on the development of adipocytes. The measurement of intracellular lipids (lipid/nuclei ratio) showed that ascorbic acid had a synergistic effect on lipogenesis but that this was dependent on BSL. Vice versa, ascorbic acid alone, i.e., without BSL, was not sufficient to promote lipogenesis. A completely new finding, however, was that ascorbic acid had an immense influence on the expression of *FABP4*. Again, the combination of BSL and ascorbic acid was shown to cause the greatest expression of *FABP4*, even in the presence of FBS. Ascorbic acid alone, however, without BSL, could not induce this effect. Thus, we assume that ascorbic acid has a positive effect on the cells because of its redox properties during the metabolically exhausting period of lipogenesis.

In **chapter 4 and 5**, we also investigated the expression pattern of Delta-like protein 1 (DLK1), which had the opposite protein expression pattern to that of FABP4. DLK1 is one of the most important regulators of homeostasis in ASC (Zwierzina et al. 2015) and is a negative regulator of adipogenesis (Mitterberger et al. 2012). DLK1 inhibits adipogenesis by modulating the MEK/ERK pathway after binding to fibronectin and therefore inhibiting PPARγ expression (Prusty et al. 2002; Hudak and Sul 2013). Thus, the protein expression pattern of FABP4 and DLK1 obviously react inversely after induction of adipogenic differentiation. As soon as FABP4 increases as a sign of adipogenesis, the ASC marker DLK1 decreases. We demonstrated exactly this behavior, i.e., immunofluorescence signals of DLK1 increased shortly after the induction of the cells but then decreased with the increase of FABP4 during adipogenesis.

Overall, we have established a highly successful, rapid, and inexpensive method of cultivating adipocytes. The production of ASC from subcutaneous tissue taken from the neck region, induced with the 30% induction medium, and differentiated in medium with BSL and ascorbic acid but without FBS gave the best results with regard to cultivation and lipogenesis during

adipogenesis. We have thus created the basis for further clinically relevant experiments using this cell model.

### Influence of insulin and magnesium on cultivated bovine adipocytes

As explained above, NEB causes cows to increase lipolysis in order to cover their energy demand. This is particularly pronounced in high-yielding cattle at the onset of lactation. A higher amount of FFA and a reduction in serum insulin concentration, insulin resistance, or both occur concomitantly with these changes (Oikawa and Oetzel 2006). The relationship between insulin action and magnesium supply has been demonstrated in human medicine, where an over-supply of magnesium significantly increases insulin sensitivity in diabetes type 2 patients and thus has a positive effect on their health (De Valk et al. 1998; De Valk 1999; Verma and Garg 2017). In contrast, little is known about such effects in cattle. A reduced magnesium serum concentration in bovines might promote ketosis during the transition period (Leno et al. 2017). In sheep, magnesium deficiency has been shown to increase lipolysis, decrease insulin responsiveness, and thus reduce glucose uptake in peripheral tissues (Matsunobu et al. 1990). An adequate magnesium supply lowers the variability in blood glucose concentrations (Ahmed et al. 2021) and the concentrations of NEFA decrease (Ataollahi et al. 2018). Magnesium as a cofactor in many enzymatic reactions (Hans et al. 2002; Jahnen-Dechent and Ketteler 2012) is essential in many metabolic processes such as glucose metabolism (Paolisso et al. 1990) or the regulation of triglycerides in blood (Kurstjens et al. 2019) and thus plays a crucial role in energy metabolism within the whole body (Saris et al. 2000).

Magnesium and insulin have an important reciprocal relationship. We can conclude that insulin is a magnesiotropic hormone and, in turn, magnesium can improve the insulin signaling pathway. An adequate magnesium supply may therefore have positive effects on insulin signaling, which in turn might reduce an exaggerated lipomobilization in cattle. However, knowledge concerning the exact interaction pathway in cattle is scarce. Therefore, **chapter 6** deals with the influence of magnesium and insulin on central metabolic functions and the expression of genes involved in magnesium homeostasis in cultured bovine adipocytes. An improved understanding of possible relationships of magnesium and insulin on lipid and carbohydrate metabolism in adipocytes might provide new therapeutic approaches for metabolic diseases in high-milk-producing cattle.

We investigated this complex interaction in bovine adipocytes cultured with the optimized protocol from the other chapters and added various concentrations of insulin and magnesium in a two-factorial design. The cells were exposed to physiological concentrations of these two factors. The effects of an undersupply and an oversupply of magnesium and insulin were

investigated concurrently. Concentrations of insulin of 25 pM and 250 pM reflect relevant plasma insulin concentrations in cows as the insulin concentration can drop from ~250 pM about three weeks before parturition to ~25 pM at two weeks after parturition (Weber et al. 2016a), a unique change compared with other mammals. Thus, the used insulin concentration of 25 pM represents an extremely reduced physiological concentration, whereas 250 pM can be considered as a high physiological concentration, and 25000 pM as supraphysiological concentration.

The physiological cytosolic concentration of free ionized magnesium in cattle is about 1 mM (Martens et al. 2018). Thus, the amount of 0.1 mM reflects extreme starvation, whereas 0.3 mM represents a low supply and 3 mM an over-supply of magnesium. The aim was to explore whether low insulin availability can be compensated by an over-supplementation of magnesium via an improvement of the insulin sensitivity in adipocytes, possibly leading to a reduction in lipolysis and lipomobilization.

Studies have shown that insulin can protect various cells from apoptosis (Tanaka et al. 1995; Kang et al. 2003). Furthermore, insulin has an adipogenic effect and exhibits protective functions on adipocytes (Loftus et al. 1998). This was demonstrated in our *in vitro* cell culture model as the lipid incorporation per cell was much higher at the high physiological insulin concentration compared to the lowest concentration of 25 pM, thereby confirming the findings of Weber et. al. (Weber et al. 2016a) and establishing the suitability of our experimental setting. The number and size of lipid vacuoles seen in the respective insulin concentrations under the microscope (at a physiological magnesium concentration of 1 mM) showed that lipid accumulation was much higher in the supraphysiological insulin concentration than in the lower concentrations (**chapter 6 figure 2**). Taken together, our results demonstrated that the availability of insulin was directly correlated with a higher accumulation of intracellular lipids after 14 and 21 days. However, the actual aim was to investigate to what extent magnesium had an effect on lipid accumulation in the interplay with insulin in cultured adipocytes. An extreme deficiency of magnesium, in this case 0.1 mM, was shown to result in a strong reduction of lipid accumulation after 14 days independent of the insulin concentration. Vice versa, a supraphysiological insulin concentration was unable to compensate for this magnesium deficiency as shown by a reduction in the amount of fat per cell across insulin concentration at 0.1 mM magnesium. In summary, a low insulin or a low magnesium concentration caused a reduction in the lipid/nuclei ratio in bovine adipocytes. This is transferable to high-milk-producing cows after parturition. A low insulin concentration or an insulin resistance provokes, together with NEB, a release of FA from adipocytes through lipolysis. This evidently natural process is essential to cover the need for energy; however, if exaggerated, serious negative effects occur with regard to the health of cows and their lactation

performance (Contreras et al. 2017b). Moreover, a recent study suggests that the concentration of FA in the blood itself strongly influences the availability of the biologically active form of magnesium. Kurstjens et al. have recently demonstrated experimentally that magnesium is directly bound by triglycerides and FA, leading to a significant reduction of the available magnesium in the blood. The authors furthermore speculate that this might be a central factor for the high prevalence of hypomagnesemia commonly observed in patients with metabolic disorders (Kurstjens et al. 2019).

We have furthermore investigated glucose uptake under our experimental conditions with the help of the glucose analog 6-NBDG. Glucose is incorporated by the insulin-dependent glucose transporter GLUT4 into adipocytes (Abe et al. 1997). The lower the basal magnesium supply, the higher is the need for insulin in order to incorporate the same amount of glucose (Kolterman et al. 1981; Kurth 2013). The 6-NBDG assay showed that the glucose uptake per cell increased with increasing insulin concentration after 14 days. Furthermore, it also showed that, after 21 days, glucose uptake was lowest in cells exposed to extreme magnesium depletion (0.1 mM). Thus, we established that insulin increases the glucose uptake and the uptake and synthesis of FA in adipocytes (in this case, BSL, acetic acid, or both serve as substrate) and thus promotes lipogenesis (Laviola et al. 2006). The finding that magnesium deficiency has a decisive influence on the insulin pathways (Paolisso et al. 1990; Gunther 2010) was also confirmed with these tests. Hypomagnesemia hinders the function of enzymes, e.g., enzymes exploiting high-energy phosphate bonds, including those in the insulin signal pathway (Paolisso et al. 1990). Moreover, a magnesium deficiency might increase the secretion of catecholamines produced by adipocytes, which induce lipolysis followed by an increase in FFA. This, however, inhibits the glucose uptake and therefore the reduction of insulin sensitivity (Volpe 2008; Gunther 2010). However, the effect of insulin in the accumulation of lipids was more evident in our Nile red studies than in the measurements of glucose uptake via the 6-NBDG assay. According to the current doctrine, GLUT4 is known to stimulate glucose uptake in an insulin-dependent manner and thus to promote FA synthesis (Winegrad et al. 1960; Huang and Czech 2007; Klip et al. 2019). In **chapter 5**, we investigated the gene and protein expression characteristics of GLUT4 in cultured bovine adipocytes. *GLUT4* expression and protein staining were detectable even in preadipocytes and became stronger with increasing differentiation. However, these results should be interpreted with caution, as large differences in expression levels were found between the individuals in our series of experiments. One reason for the variability in our GLUT4 studies (**chapter 5 and 6**) might be that the protein expression of GLUT4 in bovine adipose tissue decreases during postnatal development (Abe et al. 2001) and then once again with the onset of lactation (Jaakson et al. 2018). This in turn confirms the peculiarity of GLUT4 in bovine adipocytes in our studies and

reflects the *in vivo* situation of cows in our *in vitro* experiments in which glucose uptake in adult ruminants was shown to take place largely via the insulin-independent GLUT1 transporter (Zhao et al. 1996; Komatsu et al. 2005) with a reduction in GLUT4 transport (Hocquette et al. 2006). However, in **chapter 6**, a significant effect of magnesium on glucose uptake by adipocytes after 21 days was reported, which included the finding that a magnesium depletion to 0.1 mM resulted in significantly lower glucose uptakes at all insulin concentrations. In view of the functions of GLUT1, with its activation by phosphorylation, we can speculate that magnesium mediates glucose uptake by stimulation of the mammalian TOR complex 2 (mTORC2) (Beg et al. 2017; Liu et al. 2021). This would explain not only that magnesium is extraordinarily relevant for insulin action, which has been established many times in human medicine, e.g., in diabetes type 2 (De Valk et al. 1998; De Valk 1999; Kao et al. 1999), but also that magnesium plays an important role in transport processes that are insulin-independent, which could be, in particular, the case in ruminants.

During the transition period of cattle, the metabolic challenges and consequences are drastic and strongly interwoven between carbohydrate and lipid metabolism. Therefore, the various metabolic pathways have to be considered together, not separately. In particular, glycerol-3-phosphate dehydrogenase (GPDH) represents the link between carbohydrate and lipid metabolism (Alarcon et al. 2012). It catalyzes the reversible conversion of dihydroxyacetone phosphate and NADH from glucose metabolism to glycerol-3-phosphate for lipid synthesis (Ou et al. 2006; Mracek et al. 2013). GPDH activity is insulin-dependent, increasing with rising insulin concentration (Tu et al. 1995) and decreasing with lower insulin concentrations, as has also been shown in 3T3 cells (Guller et al. 1988). Thus, GPDH is also a marker for differentiated adipocytes (Petruschke and Hauner 1993). Our measurements of GPDH activity have shown that its activity in cultured adipocytes is significantly dependent on magnesium and insulin. However, such activity was not the highest at high insulin concentrations as previously described. The highest activity was present at the lowest insulin concentration (25 pM) and the highest magnesium concentration (3 mM). The mechanisms behind this are largely unclear. However, FA are known to reduce GPDH activity, an inhibitory effect that is associated with the increasing chain length and saturation of FA (Koekemoer et al. 1995). The unknown lipid composition of the BSL in the medium might have contributed to the reduction of GPDH activity in our assay. However, a reduction attributable to FA is unlikely to have an effect *in vivo*, as unsaturated FA and TAG, representing the main lipids during transition period in blood of cattle (Contreras et al. 2010), show little or no reducing effect on GPDH activity (Koekemoer et al. 1995). Furthermore, the highest activity of GPDH at 25 pM insulin might be explained on the basis that this corresponds to the insulin concentration postpartum in cattle and that an over-supplementation of magnesium, in times of low insulin and glucose

concentrations, might increase the synthesis of glycerol in adipocytes and thus sequestrate the high serum lipids during lipomobilization. This, in turn, would help to reduce lipid accumulation in other tissues such as liver, thereby mitigating a fundamental problem of high-yielding cattle. Nevertheless, the increase of GPDH activity in bovine adipocytes during low insulin concentrations needs further investigation and clarification.

In order to understand the influence of magnesium on the various metabolic processes in adipocytes, we further examined the so-called magnesium-responsive genes (Kolisek et al. 2019) that regulate cellular/whole body magnesium status. These genes have previously been studied in several *in vivo* (Brandao et al. 2013; Van Angelen et al. 2013) and *in vitro* (De Baaij 2015; Mastrototaro et al. 2016) experiments.

The ubiquitously expressed solute carrier family 41 member 1 (SLC41A1) is a $Na^+$ -dependent magnesium exchanger that has its highest expression levels in the heart, muscles, and kidney and that plays an important role in transmembrane magnesium transport (Kolisek et al. 2008; Kolisek et al. 2012). SLC41A1 represents the major cellular extrusion system for magnesium. This transporter is strongly dependent on the intracellular magnesium concentration and is regulated by the cAMP-dependent phosphorylation of protein kinase A (PKA), which leads to an increased excretion of magnesium, resulting in a decreased intracellular magnesium concentration (Kolisek et al. 2012). Insulin regulates the activity of SLC41A1 by activating the IR-PI3K-PDE3b pathway which, in turn, causes a decrease in cAMP, thus reducing the efflux of magnesium (Mastrototaro et al. 2015). We have shown that the expression of *SLC41A1* is positively influenced by two factors, namely insulin and magnesium. In addition, the comparison carried out for the factor insulin has demonstrated a significant difference between the highest (25000 pM) and lowest (25 pM) insulin concentrations. This can be explained on the basis that, under low extracellular magnesium concentrations, magnesium must be kept within the cells, and thus, *SLC41A1* should be expressed at a low level because it functions as an extrusion system. The inhibitory effect of insulin on SLC41A1 at higher insulin concentrations should involve an increase in the intracellular magnesium concentration, but this should be counter-regulated at very high insulin and high magnesium concentrations by an up-regulation of *SLC41A1*, as observed in our study.

Solute carrier family 41 member 3 (SLC41A3) is a sodium-dependent magnesium efflux system that resides at in the inner mitochondrial membrane (Mastrototaro et al. 2016). In our experiments with cultured bovine adipocytes, an interaction between the factors insulin and magnesium was evident for *SLC41A3* expression. Expression of the gene was lowest in medium with 0.1 mM magnesium and 250 pM insulin when compared with the other magnesium concentrations within the 250 pM insulin group. A possible explanation is that low

expression helps to conserve the mitochondrial magnesium concentration at low magnesium concentrations. To some extent, the expression patterns of *SLC41A1* and *SLC41A3* were similar. In particular, for *SLC41A3*, a magnesium deficiency together with an insufficient insulin concentration might increase its expression. This possibly leads to a stronger extrusion of magnesium from the mitochondria so that the magnesium concentration in the cytosol increases. Indeed, in previous publications, magnesium has been speculated to be mobilized from intracellular stores, e.g., mitochondria, under conditions of deficiency in order to sustain physiological magnesium concentrations within the cytosol (Kubota et al. 2005; Mastrototaro et al. 2016).

The magnesium transporter 1 (MAGT1) is expressed in all tissues in humans and promotes the transport of magnesium across the plasma membrane into the cell by means of an upregulation of expression in cases of magnesium deficiency (Zhou and Clapham 2009; Castiglioni et al. 2018). Expression of the gene in mice has been shown to be influenced by the magnesium availability in their food (Quamme 2010), and *MAGT1* has been found to be expressed in ruminal cells, the main site of magnesium resorption in adult cattle (Schweigel et al. 2008). Our experiments showed that the expression of *MAGT1* was increased at the low magnesium concentration of 0.3 mM compared with the highest concentration of 3 mM, and the lowest insulin concentration compared with the high physiological concentration of 250 pM. In a comparison of the expression of *SLC41A1* and *MAGT1* at the highest tested insulin concentration, the magnesium availability influenced the mRNA level of these genes contrariwise. This again confirms the inverse relationship of the magnesium efflux system SLC41A1 and the influx activity of MAGT1. However, recent data on MAGT1 suggest a role for the protein in the glycosylation mediated by the STT3B complex (Cherepanova and Gilmore 2016; Blommaert et al. 2019). If applicable, any putative role of MAGT1 in magnesium homeostasis seems to be more indirect than direct.

The NIPA magnesium transporter 1 (NIPA1) has been described as an electrogenic magnesium uptake mechanism that is located in many tissues, especially the brain (Goytain et al. 2007).The regulation of its expression is dependent on the extracellular magnesium concentration, with increased expression at low concentrations (Goytain et al. 2007; Kurth 2013). Knowledge about this transporter in tissues is rather scarce, and data concerning the transport characteristics of this protein have been acquired in heterologous expression systems (*Xenopus* oocytes) (Goytain et al. 2007). In our study, we found *NIPA1* to be expressed in bovine adipocytes, with a significant influence of magnesium and insulin on its expression level. Expression increased by an undersupply of magnesium (0.1 mM) compared with the highest concentration of 3 mM at the lowest insulin concentration of 25 pM; these results are consistent with those of a previous publication (Goytain et al. 2007). The expression

pattern of *NIPA1* changed at higher insulin concentrations, suggesting that, similar to SLC41A1, magnesium is retained intracellularly when insulin concentration increases.

Transient receptor potential cation channel subfamily M members 6 and 7 (TRPM6 and TRPM7) are of central importance for cellular and whole–body magnesium homeostasis, although their expression pattern differs markedly. High expression of *TRPM6* is only found in a few tissues, e.g., kidney, intestine, lung, and testis (Jang et al. 2012). Expression levels in other organs are generally low (Groenestege et al. 2006). The main function of TRPM6, therefore, appears to be the transepithelial uptake of magnesium in the intestine and resorption in the kidneys (Romani 2007). At the protein level, insulin has been shown to positively influence the cell surface expression and thereby also the transport activity of TRPM6. This mechanism is mediated by PI3K-Akt-Rac1 signaling and leads to the insertion of TRPM6-containing vesicles into the plasma membrane (Dai et al. 1999; Bezzerides et al. 2004). The effect of insulin on the mRNA expression of *TRPM6* is less well understood. In our cultured adipocytes, we found generally low expression of *TRPM6*. The expression levels differed markedly between individuals, suggesting an important role of genetic factors on the expression levels of this gene. A significant influence of magnesium and insulin on *TRPM6* expression was therefore only evident in a two-way repeated measures analysis of variance (ANOVA) in this studies. As shown in the results section of **chapter 6**, higher insulin availability decreased the expression of *TRPM6*. Moreover, the expression of *TRPM7* was not altered by insulin or magnesium. This is not unexpected, since the ubiquitously expressed magnesium channel TRPM7 is considered to be the main entry mechanism for magnesium into cells. The expression of *TRPM7* seems to be constitutive and, in contrast to *TRPM6*, is not influenced by the magnesium content of the diet (Groenestege et al. 2006). TRPM7 channels have been shown to regulate adipogenesis, especially in the adipocyte precursor cell line 3T3-L1. A deactivation of TRPM7 decreases the differentiation of these cells (Chen et al. 2014). However, since we performed the studies on differentiated adipocytes, the expression patterns of *TRPM7* were constantly independent of insulin or magnesium.

The magnesium transporter cyclin and CBS domain divalent metal cation transport mediator 2 (CNNM2) is ubiquitously expressed and is highly abundant in kidney and brain, although its exact function has not yet been conclusively delineated (Sponder et al. 2016). *CNNM2* showed no significant changes in its expression in dependence on insulin and magnesium availability. Furthermore, the expression of *CNNM2* was generally low in all analyzed samples suggesting that *CNNM2* does not play a prominent role in the magnesium homeostasis of adipocytes.

The solute carrier family 41 member 2 (SLC41A2) might also function as a magnesium transporter but is also permeable to other cations, such as iron, cobalt, or manganese (Brandao et al. 2013). This transporter was examined for completeness and, like *CNNM2*, showed no significant changes in expression in bovine adipocytes.

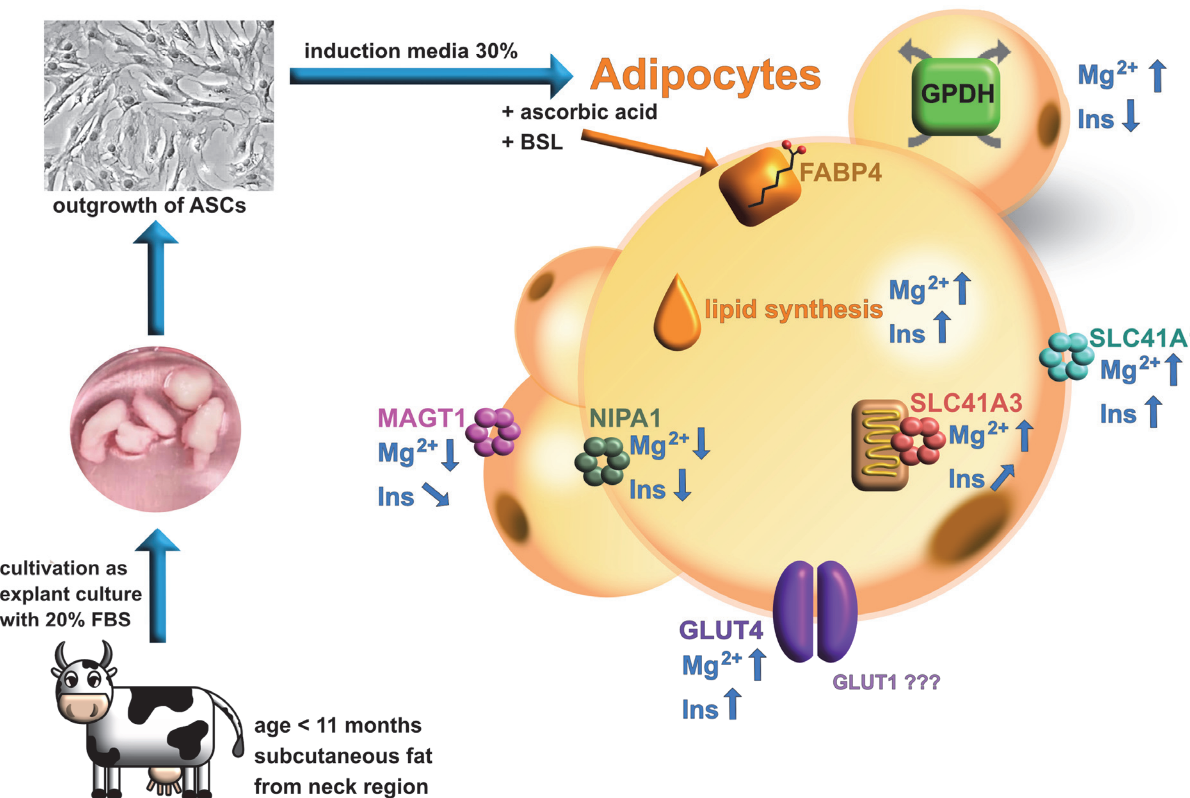


**Figure 2: Summary presentation of the main results of the influence of insulin and magnesium on cultured bovine adipocytes.**

Subcutaneous adipose tissue from the neck region of calves less than 11 months old showed the best characteristics for culturing. This was performed as an explant culture with the addition of 20% FBS. After ASC grew out of the explants, the cells were induced with the 30% induction medium. Adipocytes were obtained that responded positively to ascorbic acid and BSL addition by increasing FABP4 expression. The influence of magnesium and insulin on lipid synthesis, GPDH activity, glucose uptake, and expression of magnesium responsive genes is shown with arrows. The activity or expression increased (↑) or decreased (↓) with increasing magnesium (0.1, 0.3, 1, 3 mM) or insulin (25 pM, 250 pM, 25000 pM) concentrations. Variable responses are symbolized by a descending or ascending arrow (own graphic created for this work 2021).

In conclusion, as stated in **chapter 6**, the influence of insulin and magnesium on adult adipocytes can be successfully investigated by using our protocol of differentiation. Magnesium and insulin increase adipocyte differentiation and lipogenesis, which can be seen in measurements of lipid accumulation in cells. The effect of insulin on glucose uptake was not as clear as expected, suggesting glucose uptake via the insulin-independent transporter

GLUT1, which may play an important role in ruminants. Nevertheless, the effect of magnesium was clearly visible in glucose uptake and GPDH activity. Furthermore, the magnesium genes *NIPA1* and *SLC41A3* were shown to be upregulated under low insulin concentrations (**Figure 2**). In times of low glucose and low insulin concentrations, as is the case during the transition period in high-milk-producing cattle, an over-supplementation of magnesium might positively influence the metabolic processes to the extent that NEFA, circulating in the blood are sequestered in adipocytes during lipomobilization and not in organs such as the liver, as is the case in the fatty liver syndrome of cattle. The latter syndrome is a large problem affecting the health status and performance in cows.

## Outlook and potential improvements

Overall, the protocols presented here can successfully be used to establish the cultivation and differentiation conditions for bovine adipocytes in order to examine the possible influences of magnesium and insulin on certain metabolites and to perform assays and investigations on cultured adult bovine adipocytes. Nevertheless, problems with the detachment of the cells from the cell culture dishes occurred upon long duration of experiments, particularly in media that provided conditions for increased lipogenesis. This was especially evident as the differentiation of adipocytes progressed. The unfortunate consequence of strong lipid accumulation was that the adipocytes detached completely from the bottom of the culture flask. These fat-filled floating adipocytes could not be included in the measurements (e.g., Nile red staining and measurement, 6-NBDG or GDPH assay) for which adherent cells were required. The floating and clumping of adipocytes with high lipid accumulation, as observed in our study, limit the accessibility of these cells for several assay techniques. This disadvantage was particularly pronounced in our experimental setup in which adipocytes had been cultured for extended periods. To address this problem, we attempted, in **chapter 5**, to test various coating reagents for cell culture flasks that had previously been used in studies on MSC, e.g., poly-L-lysine (Lu et al. 2009), collagen (Somaiah et al. 2015), and gelatin (Su et al. 2014). We showed that, in cell culture flasks coated with gelatin and poly-l-lysine, significantly higher nuclei indices could be achieved than in flasks coated with collagen or flasks that were not coated at all. Thus, cell adhesion was better maintained by an improved coating of the growth surface, even if the intracellular fat droplets with their lower density still promoted some cell detachment. Nevertheless, further improvements are desirable, especially if trials are long and lipogenesis, i.e., the formation of fat vacuoles, is extensive. In the future, an interesting possibility might be the establishment of 3D cultures with various matrix components that completely envelope the cells without affecting their properties with regard to their metabolic processes or gene expression, but that prevent the cells from detaching (Emont et al. 2015; Louis et al. 2017).

This could enable long-term cell experiments without the loss of adipocytes because of floating.

Although our investigations with regard to adapted induction and differentiation media were successful, the culture conditions merely mimicked those *in vivo*. The communication between the various cell types in the cell association of adipose tissue is highly complex and dynamic and poses a challenge to the cultivation of adipocytes. In order to enable a particularly authentic growth of adipocytes, the use of a co-culture model is certainly of great interest. This involves the induction of preadipocytes in the presence of mature adipocytes in special inserts in cell culture plates, providing indirect contact between preadipocytes and mature adipocytes via the medium. Paracrine signals and growth hormones that are secreted by adult adipocytes are known to increase differentiation (Considine et al. 1996). Strieder-Barboza et al. were able to establish such a co-culture model with bovine adipocytes and have shown that induction occurs more rapid in the co-culture model compared with a conventional protocol, namely in 7 days rather than 14 days and with a much more realistic, natural, and physiological environment for the adipocytes (Strieder-Barboza et al. 2019). This approach is of interest for future studies and for the cultivation of bovine adipocytes. However, for this purpose, adipose tissue has to be readily available for the cultivation of preadipocytes, e.g., via biopsies from living cows, because mature adipocytes from adipose tissue need to be added some days later. Nevertheless, such an approach would be a time-efficient solution for culturing bovine adipocytes as more lengthy preliminary phases are no longer required.

Another interesting aspect that needs to be investigated in more detail is the use of acetic acid in the differentiation medium of adipocytes. Unlike adipose tissue from other mammals, acetic acid and not glucose is the basic source for FA synthesis in bovine adipocytes (Vernon 1980). In 3T3-L1 cells, acetic acid has been shown to accelerate fat synthesis and the expression of enzymes for FA metabolism such as *FABP4*, *FAS*, or *LPL* (Yu et al. 2018). In **chapter 5**, we investigated the extent to which various concentrations of glucose and acetic acid had an effect on the lipogenesis of cultured adipocytes. We found that a reduction of the glucose concentration from 25 mM to 10 mM did not result in a significant effect within the various acetic acid concentrations. Increasing the acetic acid concentration to 10 mM or 20 mM produced no significant effect on lipid storage in the adipocytes, but a slight numerical increase in the lipid/nuclei ratio was detected. Hence, we can speculate that acetic acid has a positive effect on lipid accumulation in bovine adipocytes, although this needs to be investigated further, especially via a more comprehensive titration approach in order to identify the most suitable acetic acid concentration. In our pilot experiments, 20 mM acetic acid appeared to be too high as indicated by the finding that the cell number (nuclei index) decreased between 10

mM and 20 mM. Thus the concentrations of 10 mM and 20 mM acetic acid might both be too high for cultured adipocytes, but this requires additional studies.

The presented good results for the cultivation of adipose cells should facilitate further progress in adipocyte research and allow interesting questions regarding adipose tissue physiology to be answered.

# Chapter 8: Summary


### "Influence of magnesium on endocrine regulation and energy metabolism in bovine adipocytes"

High performance cattle go through a tremendous increase in energy requirements from about 3 weeks before to 3 weeks after calving. Feed intake at the onset of lactation is insufficient, leading to a negative energy balance (NEB) which is compensated for by the depletion of fat reserves in the body. Furthermore, a physiological insulin deficiency or insulin resistance or both occur in tissues such as the liver, muscle, or adipose tissue in order to supply insulin-independent tissues such as the mammary gland with sufficient glucose for lactose production. This state promotes metabolic dysfunctions with diseases like ketosis, milk fever, or inflammatory reactions like mastitis. As the main cells engaged in fat metabolism of cattle, adipocytes not only play an essential role in energy balance, but also have a whole-body influence on the health of cattle.

Magnesium as the second most abundant intracellular cation plays a prominent role in the insulin signaling pathway. Magnesium homeostasis is tightly regulated with a reciprocal relationship between insulin and magnesium. The main aim of the present study was to investigate and to understand the interaction between insulin and magnesium on bovine adipocytes, with the hypothesis that magnesium could have positive effects on animal health by improving the insulin sensitivity of adipocytes. To facilitate this research, a cell culture protocol for preadipocyte differentiation into bovine adipocytes was established.

Mesenchymal stem cells derived from subcutaneous adipose tissue in the neck region of calves were shown to yield large numbers of preadipocytes when cultured as explants. Cells showed the expression of stem cell markers, namely CD73, CD90, and CD105.in immunohistochemical and qRT-PCR studies. A higher expression of stem cell markers in preadipocytes was dependent on fetal bovine serum (FBS). In contrast, bovine serum lipids (BSL) increased adipocyte differentiation, with downregulation of stem cell markers and upregulation of adipocyte markers, e.g., *FABP4*. Thus, FBS is essential for the cultivation of mesenchymal stem cells by promoting cell replication and the expression of stem cell markers, whereas BSL counteracts this and promotes differentiation.

Furthermore, it could be shown that important media additives for induction, e.g., dexamethasone, insulin, rosiglitazone, IBMX, and biotin, could be reduced to 30% of the original value derived from literature without reducing induction performance. The addition of ascorbic acid as a relevant supplement for collagen synthesis and as an antioxidant showed that, together with BSL, differentiation was most successful under these conditions. Ascorbic acid had outstanding effects on the expression of the adipocyte marker *FABP4* and on *LPS* and the protein expression of FAS.

During differentiation adipocytes increasingly formed intracellular fat vacuoles, as evidenced by Nile red staining. Because of lower density of the fat that they contained, loss of cells from the bottom of the cell culture flasks occurred. Coating of culture plates with poly-L-lysine or gelatin caused significantly better adherent cell numbers than flasks with collagen coating or without coating.

To investigate the influence and interplay of magnesium and insulin on bovine adipocytes, cells were exposed to various magnesium and insulin concentrations in a two-factorial design. Lipid accumulation increased with increasing insulin concentrations, veryfying that insulin acts as an adipogenic and protective factor for adipocytes. Severe magnesium deficiency (0.1 mM) led to lower lipogenesis of cells, independently of insulin concentration. A similar pattern was seen for glucose uptake where insulin also had a promoting effect, but magnesium starvation at 0.1 mM reduced glucose uptake. Nevertheless, this effect was not as pronounced as in lipid accumulation; hence, we can assume that insulin-independent glucose uptake mechanisms are also present, for example, via the glucose transporter GLUT1, which is also influenced by magnesium. To investigate the interplay of glucose and lipid metabolism further, a glycerol-3-phosphate dehydrogenase (GPDH) assay was performed. Contrary to expectations, the highest activity of GPDH was detectable in cells grown at a low physiological insulin concentration of 25 pM and in those cultured at the highest magnesium concentration of 3 mM. Considering that cattle have low insulin and glucose concentrations in the blood after calving, the present results suggest that a high supply of magnesium might promote an increased incorporation of glycerol into fat cells *in vivo*, thereby, decreasing the danger of fatty liver syndrome.

In the last part of this work, the so-called magnesium-responsive genes were investigated by RT-qPCRs. The transporters SLC41A2, TRPM6, TRPM7, and CNNM2 showed no significant changes in their expression patterns in bovine adipocytes and thus were not affected by the concentrations of insulin or magnesium. While the expression of *SLC41A1* was found to be decreased at low magnesium concentrations, presumably in order to keep the intracellular magnesium content constant. Furthermore, an inhibitory effect of insulin was shown.

*SLC41A3* showed a reduced expression in cells in culture medium with 250 pM insulin, similar to *SLC41A1*, and in low magnesium concentrations. This reduction in expression might keep mitochondrial magnesium concentration constant at low magnesium availability. The magnesium transporter *MAGT1*, which transports magnesium into the cell, was expressed to a greater extent at low magnesium concentrations, showing an opposite and traceable expression pattern to *SLC41A1*. *NIPA1* also showed upregulation at low magnesium concentrations, although this effect was neutralized at high insulin concentrations. These results show that magnesium has an essential influence on insulin signalling pathways and thus fat metabolism in bovine adipocytes providing the foundation for future investigations for potential therapeutic approaches to prevent excessive lipomobilization together with possible associated diseases in cattle.

**Zusammenfassung der Dissertation "Einfluss von Magnesium auf die endokrine Regulation und den Energiemetabolismus in bovinen Adipozyten"**

Hochleistungsrinder durchleben in der Zeit von etwa 3 Wochen vor bis 3 Wochen nach der Kalbung einen enormen Anstieg des Energiebedarfs. Die Futteraufnahme ist zu Beginn der Laktation unzureichend, was zu einer negativen Energiebilanz (NEB) führt, die kompensiert wird, indem Fettreserven des Körpers abgebaut werden. Darüber hinaus kommt es in Geweben wie der Leber, der Muskulatur oder dem Fettgewebe zu einem physiologischen Insulinmangel und/oder einer Insulinresistenz, um insulinunabhängige Gewebe, wie die Milchdrüse, mit ausreichend Glukose für die Laktoseproduktion zu versorgen. Dieser Zustand der negativen Energiebilanz kann zu Stoffwechselstörungen mit nachfolgenden Krankheiten, etwa Ketose, Milchfieber oder entzündlichen Erkrankungen wie Mastitis oder Metritis führen. Adipozyten spielen im Fettstoffwechsels bei Rindern nicht nur eine essentielle Rolle im Energiehaushalt, sondern beeinflussen multifaktoriell ganzkörperlich den Gesundheitszustand des Rindes.

Magnesium, das zweithäufigste intrazelluläre Kation, spielt eine herausragende Rolle im Insulinsignalweg. Die Magnesiumhomöostase ist genauestens reguliert, wobei es ein wechselseitiges Verhältnis zwischen Insulin und Magnesium gibt. Ziel der vorliegenden Studie war es, diese Wechselwirkung auf Adipozyten von Rindern zu untersuchen und zu verstehen. Magnesium könnte sich möglicherweise positiv auf die Tiergesundheit auswirken, indem die Insulinempfindlichkeit der Fettzellen verbessert wird.

Es zeigte sich, dass mesenchymale Stammzellen von Kälbern aus subkutanem Fettgewebe der Nackenregion eine große Anzahl von Präadipozyten liefern, wenn man diese als Explantatkultur kultiviert. Weiterhin exprimierten diese Zellen Stammzellmarker wie CD73, CD90 und CD105, die durch immunhistochemische und qRT-PCR-Studien ebenfalls nachgewiesen werden konnten. Eine erhöhte Expression von Stammzellmarkern in Präadipozyten war hierbei abhängig vom fötalen Kälberserum (FKS), wohingegen bovine Serumlipide (BSL) die Differenzierung der Adipozyten steigerte, sodass die Expression von Stammzellmarkern herunterreguliert wurde, wohingegen Adipozytenmarker, z.B. FABP4, vermehrt exprimiert wurden. Somit ist FBS für die Kultivierung von mesenchymalen Stammzellen unerlässlich, da es die Zellreplikation und die Expression von Stammzellmarkern fördert, während BSL dem entgegenwirkt und die Differenzierung fördert.

Darüber hinaus konnte gezeigt werden, dass wichtige Medienzusätze für die Induktion, z.B. Dexamethason, Insulin, Rosiglitazon, IBMX und Biotin, auf 30% der aus der Literatur extrahierten Werte reduziert werden konnten, ohne die Induktionsleistung zu verringern. Die Zugabe von Ascorbinsäure als relevanter Zusatz für die Kollagensynthese und als Antioxidans zeigte, dass zusammen mit BSL die Differenzierung unter diesen Bedingungen am

erfolgreichsten stattfand. Ascorbinsäure hatte herausragende Effekte auf die Expression des Adipozytenmarkers *FABP4* sowie auf *LPS* und die Proteinexpression von FAS, die ähnliche Expressionsmuster zeigten.

Da die Adipozyten während der Differenzierung zunehmend intrazelluläre Fettvakuolen bilden, was anhand von Nile-red Färbungen nachgewiesen werden konnte, trieben diese aufgrund der geringeren Dichte des Fettes nach oben. Ein Verlust der Zellen vom Boden der Zellkulturflaschen war die Folge. Eine Beschichtung mit Poly-L-Lysin oder Gelatine verbesserte die Zahlen adhärenter Zellen im Vergleich mit Flaschen ohne Beschichtung oder mit einer Beschichtung aus Kollagen.

Um den Einfluss und das Zusammenspiel von Magnesium und Insulin an bovinen Adipozyten zu untersuchen, wurden die Zellen in einem zweifaktoriellen Design verschiedenen Magnesium- und Insulinkonzentrationen ausgesetzt. Dabei konnte gezeigt werden, dass die intrazelluläre Lipidakkumulation mit steigenden Insulinkonzentration steigt. Starke Magnesium-Mangelbedingungen von 0,1 mM zeigten eine geringere Lipogenese der Adipozyten, unabhängig von der Insulinkonzentration. Ein ähnliches Muster zeigte sich bei der Glukoseaufnahme durch die Zellen, wo Insulin ebenfalls eine fördernde Wirkung hatte, aber ein Magnesiummangel von 0,1 mM die Glukoseaufnahme verringerte. Allerdings war dieser Effekt nicht so stark ausgeprägt wie bei der Lipidakkumulation, sodass davon auszugehen ist, dass auch insulinunabhängige Mechanismen der Glukoseaufnahme vorhanden sind, etwa der Glukosetransporter GLUT1, der ebenfalls Magnesiumabhängig ist.

Um das Zusammenspiel von Glukose- und Lipidstoffwechsel weiter zu untersuchen, wurde ein Glycerin-3-Phosphat-Dehydrogenase (GPDH)-Test durchgeführt. Entgegen den Erwartungen war die höchste Aktivität von GPDH in Zellen nachweisbar, die unter der niedrigen physiologischen Insulinkonzentration von 25 pM und der höchsten Magnesiumkonzentration von 3 mM kultiviert wurden. Berücksichtigt man, dass Rinder nach dem Kalben niedrige Insulin- und Glukosekonzentrationen im Blut haben, könnte eine hohe Magnesiumkonzentration *in vivo* den vermehrten Einbau von Glycerin in Fettzellen fördern und dadurch die Leber besser vor einem Fettlebersyndrom schützen.

Im letzten Teil dieser Arbeit wurden die so genannten magnesium-sensitiven Gene mittels RT-qPCRs untersucht. Die Gene *SLC41A2*, *TRPM6*, *TRPM7* und *CNNM2* zeigten keine signifikanten Änderungen in ihren Expressionsmustern in bovinen Adipozyten und wurden somit weder von der Insulin- noch der Magnesiumkonzentrationen beeinflusst. Jedoch war die Expression von *SLC41A1* bei niedrigen Magnesiumkonzentrationen verringert, vermutlich um den intrazellulären Magnesiumgehalt konstant zu halten. Des Weiteren zeigte sich ein inhibitorischer Effekt von Insulin auf dessen Expression. *SLC41A3* zeigte eine Reduktion der Expression bei 250 pM Insulin und niedrigen Magnesiumkonzentrationen; möglicherweise, um die mitochondriale Magnesiumkonzentration bei geringer Verfügbarkeit konstant zu halten.

Der Magnesiumtransporter *MAGT1*, der Magnesium in das Zellinnere transportiert, wurde bei niedrigen Magnesiumkonzentrationen stärker exprimiert und zeigte ein entgegengesetztes Expressionsmuster zu *SLC41A1*. *NIPA1* zeigte ebenfalls eine Hochregulierung bei niedrigen Magnesiumkonzentrationen, wobei dieser Effekt bei hohen Insulinkonzentrationen neutralisiert worden ist. Diese Ergebnisse zeigen, dass Magnesium einen wesentlichen Einfluss auf die Insulinsignalwege und damit auf den Fettstoffwechsel in bovinen Adipozyten hat. Dies könnte die Grundlage für zukünftige therapeutische Ansätze zur Verhinderung einer übermäßigen Lipomobilisierung und der damit einhergehenden Krankheiten bei Rindern schaffen.

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## List of publications

### Publications (peer reviewed)

- Rashid, U., Yousaf, A., Yaqoob, M., Saba, E., Moaeen-ud-Din, M., Waseem, S., **Becker, S.K.**, Sponder, G., Aschenbach, J.R., Sandhu, M.S. **(2021).** Characterization and differentiation potential of mesenchymal stem cells isolated from multiple canine adipose tissue sources.
  BMC Veterinary Research 17, 388. doi: 10.1186/s12917-021-03100-8.

- **Becker, S.K.**, Sponder, G., Sandhu, M.A., Trappe, S., Kolisek, M., Aschenbach, J.R. **(2021)**. The combined influence of magnesium and insulin on central metabolic functions and expression of genes involved in magnesium homeostasis of cultured bovine adipocytes. International Journal of Molecular Sciences. 22(11): 5897. doi: 10.3390/ijms22115897.

- **Jurek, S.**, Sandhu, M.A., Trappe, S., Bermúdez-Peña, M.C., Kolisek, M., Sponder, G., Aschenbach, J.R. **(2020)**. Optimizing adipogenic transdifferentiation of bovine mesenchymal stem cells: a prominent role of ascorbic acid in *FABP4* induction. Adipocyte. 9(1):35-50. doi: 10.1080/21623945.2020.1720480.

- Sandhu, M.A., **Jurek, S.**, Trappe, S., Kolisek, M., Sponder, G., Aschenbach, J.R. **(2017)**. Influence of bovine serum lipids and fetal bovine serum on the expression of cell surface markers in cultured bovine preadipocytes. Cells Tissues Organs. 204(1):13-24. doi: 10.1159/000472708.

### Abstracts in proceedings & participation in conferences

- **Becker, S.**; Aschenbach, J. R.; Sponder, G. **(2021)**:
  Insulin and Mg2+ influence the expression of Mg transporters and of genes with central metabolic functions in the bovine liver cell line BFH12
  75. digitale Tagung der Gesellschaft für Ernährungsphysiologie
  Berlin – 16.03.-18.03.2021.
  In: Gesellschaft für Ernährungsphysiologie (Hrsg.) Frankfurt am Main (Germany): DLG-Verlag GmbH. Proceedings of the Society of Nutrition Physiology : Berichte der Gesellschaft für Ernährungsphysiologie; volume 30 (2021), S. 115
  ISBN: 978-3-7690-4114-9

- **Jurek, S.K.**; Aschenbach, J. R.; Martens, H.; Sponder, G. **(2019)**:
  Differential expression of genes associated with Mg2+ homeostasis in dairy cows with high-and low back fat mobilization.

73. Tagung der Gesellschaft für Ernährungsphysiologie
Göttingen – 13.03.-15.03.2019.
In: Gesellschaft für Ernährungsphysiologie (Hrsg.) Frankfurt am Main (Germany): DLG-Verlag GmbH. Proceedings of the Society of Nutrition Physiology : Berichte der Gesellschaft für Ernährungsphysiologie; volume 28 (2019), S. 30
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- **Jurek, S.K.**; Sandhu, M. A.; Sponder, G.; Trappe, S.; Kolisek, M.; Aschenbach, J. R. **(2019)**:
  Magnesium and insulin stimulates the glucose uptake in differentiated bovine adipocytes.
  XV International Magnesium Symposium Magnesium in Health and Disease
  NIH National Instituts of Health, Bethesda, USA – 20.03.-22.03.2019.
  In: XV International Magnesium Symposium Magnesium in Health and Disease Program and Abstracts – National Institutes of Health (Hrsg.)
  Bethesda, S. 83

- **Jurek, S.**; Sadhu, M. A.; Trappe, S.; Kolisek, M.; Sponder, G.; Aschenbach, J. R. **(2019)**:
  Insulin and magnesium increase the activity of GPDH in bovine adipocytes.
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  Leipzig – 03.09.-06.09.2019.
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- **Jurek, S.**; Sandhu, M. A.; Aschenbach, J. R.; Kolisek, M.; Sponder, G.; Trappe, S. **(2018)**:
  Role of ascorbic acid, serum lipids and acetic acid during transdifferentiation of bovine pre-adipocytes to adipocytes.
  23. Tagung der DVG-Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft
  Wien – 21.02.-23.02.2018.
  In: 23. Tagung der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft : PROGRAMM & ABSTRACTS – Veterinärmedizinische Universität Wien (Hrsg.) S. 81

- **Jurek, S.K.**; Sandhu, M. A.; Trappe, S.; Kolisek, M.; Sponder, G.; Aschenbach, J. R. **(2018)**:
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- **Jurek, S.**; Sandhu, M. A.; Aschenbach, J. R. **(2017)**:
  Influence of ascorbic acid, serum lipids and acetic acid on the adipogenic potential of bovine adipose stem cells.
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  Leipzig, S. 58–59

- Sandhu, M. A.; **Jurek, S.K.**; Trappe, S.; Kolisek, M.; Aschenbach, J. R. **(2017)**:
  Role of glucose and acetic acid in differentiation of bovine pre-adipocytes into mature adipocytes.
  71. Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE)
  Göttingen – 14.03.-16.03.2017.
  In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.) Frankfurt am Main: DLG Verlag GmbH. Gesellschaft für Ernährungsphysiologie: Proceedings of the Society of Nutrition Physiology; 26, S. 157
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- Sandhu, M.A.; **Jurek, S.K.**; Trappe, S.; Bermudez, P.; Kolisek, M.; Müller, K.E.; Aschenbach, J.R. **(2016)**:
  Optimization of culture media supplements for bovine preadipocyte transformation to adipocytes.
  22. Tagung der DVG-Fachgruppe "Physiologie und Biochemie"
  Berlin – 30.03.-01.04.2016.
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## Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

### Declaration of independence

I hereby confirm that I have written this thesis independently. I certify that I have used only the sources and helps indicated.

Berlin, 22.09.2022
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