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Adiponectin in Cattle: Profiling of molecular weight patterns in different body fluids at different physiological states and assessment of adiponectin's effects on lymphocytes

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Adiponectin in cattle: Profiling of the molecular weight patterns in different body fluids at different physiological states and assessment of adiponectin's effects on lymphocytes

Adiponectin (AdipoQ), one of the most abundant adipokines found in circulation exerts various metabolic functions, e.g. improving insulin sensitivity and ameliorating tissue inflammation. It is secreted in different molecular weight (MW) forms: a low molecular weight (LMW) trimer, a middle molecular weight (MMW) hexamer and a high molecular weight (HMW) form which is built of 12 to 18 monomers. Dairy cows undergo various metabolic changes in the time from late pregnancy to early lactation. This causes a mobilization of body reserves which may lead to a higher risk for infectious diseases and possible problems in fertility later. The aims of this thesis were (1) to establish a semi-quantitative Western blot to estimate AdipoQ concentrations in serum and milk of lactating dairy cows; (2) to develop a semi-native Western blot to differentiate AdipoQ MW patterns in several bovine body fluids and tissues. (3) to estimate potential influences of AdipoQ on lymphocyte function; for this purpose AdipoQ was recombinantly expressed in *Escherichia coli*.

First, the AdipoQ serum concentration in late pregnancy and the entire lactation as well as the concentrations in milk from d 1 to d 24 in lactation were estimated. Subsequently, a profile of the AdipoQ MW forms in serum and milk of dairy cows at different time points in lactation was generated. Furthermore, the MW patterns of AdipoQ in two different adipose tissue (AT) depots (visceral and subcutaneous) at three different days (1, 42, and 105) after parturition were investigated. In addition the MW patterns of AdipoQ in the mammary gland were shown. The AdipoQ MW forms in cerebrospinal fluid (CSF) and corresponding serum of transition cows were characterized. Moreover the AdipoQ MW patterns in other *Bovidae*, i.e.Yak, Bison and Water buffalo were characterized. As body fluids in relation to reproduction we investigated the AdipoQ MW patterns in allantoic fluid (AF) and corresponding maternal serum. In addition the AdipoQ concentrations and MW patterns in seminal plasma (SP) of bulls and follicular fluid (FF) of heifers were evaluated. Independent of the MW patterns, the functional effect of recombinant AdipoQ on lymphocyte proliferation was studied.

Adiponectin concentration in serum and milk showed an inverse course. Serum AdipoQ decreased until parturition and increased in early lactation, whereas AdipoQ concentration in milk was highest at the onset of lactation and decreased reaching a nadir in the first week of lactation. The changes in circulating AdipoQ are probably related with the hormonal changes associated with parturition. The MW patterns of serum and milk showed a prominent MMW band and a faint HMW band. In contrast to the MW patterns observed in humans we speculate that the MMW form of AdipoQ might be the most abundant one in cattle; in Yak, Bison and Water buffalo, the MMW AdipoQ was also the most prominent one. Different AT and mammary gland homogenates showed no differences in molecular weight pattern of AdipoQ. At each stage of lactation the HMW and the MMW band was detectable. CSF and serum samples of individual days in transition period showed no apparent differences in the MW pattern of AdipoQ. The AdipoQ MW pattern in AF was different to the AdipoQ MW pattern seen in serum before. AdipoQ was mainly detected as the HMW form, which might indicate that AF AdipoQ is not derived from circulation and might be of fetal origin. In bulls AdipoQ serum concentrations correlated with the ones in SP and the MW distribution was mainly the same. AdipoQ MW pattern in FF of heifers was different to the serum MW pattern; The HMW band was virtually absent in FF independent of the stage of the estrous cycle. Recombinant AdipoQ reduced mitogen induced lymphocyte proliferation which indicates that AdipoQ might be involved in the immune suppression. The results of this thesis provide AdipoQ profiles in several bovine body fluids. The physiological function of the individual AdipoQ isoforms needs to be further investigated.

Adiponektin beim Rind: Darstellung der Molekulargewichtsformen in unterschiedlichen Körperflüssigkeiten in verschiedenen physiologischen Zuständen und Ermittlung des Adiponectineffekts auf Lymphozyten

Adiponektin (AdipoQ) ist eines der am häufigsten in der Zirkulation vorkommenden Adipokine. Es beeinflusst verschiedene metabolische Prozesse und trägt zur Verbesserung der Insulinsensitiviät und der Eindämmung von Entzündungen im Gewebe bei. Die Sekretion erfolgt in drei unterschiedlichen Molekulargewichtsformen (MW): als Trimer in der niedermolekularen Form (low molecular weight, LMW), als Hexamer in der mittleren Molekularform (middle molecular weight, MMW), sowie als multimere hochmolekulare Form (high molecular weight, HMW), bestehend aus 12-18 Monomeren. Milchkühe sind in der Zeit der späten Trächtigkeit und frühen Laktation vielen metabolischen Veränderungen ausgesetzt. Die Mobilisierung von Körperreserven kann zu einem erhöhten Risiko für Infektionskrankheiten führen und beeinflusst möglicherweise auch die spätere Fortpflanzungsleistung. Ziel dieser Arbeit war (1) die Etablierung eines semi-quantitativen Western Blots zur Bestimmung der AdipoQ-Konzentration in Serum und Milch von Milchkühen im geburtsnahen Zeitraum. Zusätzlich erfolgte (2) die Entwicklung eines semi-nativen Western Blots, um die unterschiedlichen MW von AdipoQ in verschiedenen Köperflüssigkeiten und Geweben zu charakterisieren. Desweiteren wurden (3) mögliche Auswirkungen von AdipoQ auf die Funktionsfähigkeit von Lymphozyten untersucht. Hierzu wurde AdipoQ rekombinant in *Eschericha coli* hergestellt. Im ersten Schritt wurde die AdipoQ-Konzentration in Serum von Milchkühen während der späten Trächtigkeit sowie im Verlauf der Laktation bestimmt, anschließend in Milch im Zeitraum vom 1. bis zum 24. Tag der Laktation. Im Anschluss erfolgte die Erstellung eines Molekulargewichtprofils in Serum und Milch von Milchkühen in der frühen und mittleren Laktation. Darüber hinaus wurden die MW von AdipoQ in zwei verschiedenen Fettgeweben (adipose tissue, AT) (viszeral und subkutan) an Tag 1, 42 und 105 der Laktation, sowie in der Milchdrüse gezeigt. Weiterhin wurde das MW-Profil von AdipoQ in zerebrospinaler Flüssigkeit (cerebrospinal fluid, CSF) und korrespondierendem Serum von Milchkühen im peripartalen Zeitraum untersucht. In einem weiteren Schritt erfolgte die Ermittlung der AdipoQ-Konzentration und des MW-Profils in Serum und Reproduktionsflüssigkeiten von Rindern; Seminalflüssigkeit (seminal plasma, SP) von Bullen, sowie Follikelflüssigkeit (folicular fluid, FF) und Fruchtwasser (alantois fluid, AF) von Färsen. Überdies konnten die MW von AdipoQ auch in artverwandten Spezies der Rinder (Yak, Bison, Wasserbüffel) dargestellt werden. Unabhängig vom MW wurden die Auswirkungen von AdipoQ auf die Proliferation von Lymphozyten bestimmt. Die Serum- und Milch-AdipoQ-Konzentrationen verliefen gegenläufig, im Serum sank die Konzentrationen bis zur Geburt und stieg danach wieder an. In Milch sank die AdipoQ-Konzentration im Verlauf der ersten Laktationswoche wieder. Die Veränderungen der AdipoQ Konzentrationen stehen vermutlich in Verbindung mit den hormonellen Veränderungen im geburtsnahen Zeitraum.

Das Profil der AdipoQ-MW in Serum und Milch zeigte eine prominente MMW-Bande und eine feine HMW Bande. Anders als beim Menschen könnte beim Rind die MMW die vorherrschende AdipoQ-Form darstellen. Auch in Yak, Bison und Wasserbüffel war die MMW die prominenteste Bande. In den verschiedenen AT und der Milchdrüse konnte kein Unterschied im AdipoQ-MW bestimmt werden. Zu jedem Zeitpunkt in der Laktation konnte eine MMW und eine HMW Bande detektiert werden. CSF und Serum von unterschiedlichen Zeitpunkten in der Übergangsphase zeigten keinen Unterschied in den MW von AdipoQ. In AF konnte nur eine HMW-Bande nachgewiesen werden nicht wie im Serum, was dafür spricht, dass AF-AdipoQ nicht aus der Zirkulation kommt und möglicherweise fötalen Ursprungs ist. In Bullen korrelierte die AdipoQ-Serumkonzentration mit der im SP und auch die MW-Formen waren sich ähnlich. Die MW-Verteilung in FF und Serum war unterschiedlich, in FF war nur die MMW Bande zu finden, unabhängig vom Zeitpunkt im Zyklus. Rekombinant produziertes AdipoQ war in der Lage die Lymphozyten-proliferation zu senken, was darauf hindeuten könnte, dass AdipoQ Einfluss auf eine Immunsuppression haben könnte. Die Ergebnisse dieser Dissertation geben einen Überblick über die AdipoQ MW-Profile in unterschiedlichen bovinen Körperflüssigkeiten und Geweben.

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List of abbreviations

a.p.	ante partum
AdipoQ	adiponectin
AdipoR1 and AdipoR2	adiponectin receptor 1 and 2
AF	allantoic fluid
AMPK	adenosine monophosphate-activated protein kinase
APPL1	adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif
AT	adipose tissue
BHB	beta-hydroxybutyrate
cDNA	copy deoxyribonucleic acid
Con A	concanavalin A
CSF	cerebrospinal fluid
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E.coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
Ero1-Lα	endoplasmic reticulum oxidoreductase 1-Lα
ERp44	endoplasmic reticulum protein of 44 kDa
EU	endotoxin units
FCS	fetal calf serum
FF	follicular fluid
HMW	high molecular weight
HRP	horseradish peroxidase
IFNγ	interferon-γ
IgM	immune globulin M
IL-10	interleukin-10
LAL	limulus amebocyte lysate
LB-medium	Luria Bertani medium

LEW	lyses- equilibration- washing
LMW	low molecular weight
LPS	lipopolysaccharid
LSM	lymphocyte separation medium
MMW	middle molecular weight
MP	multiparous
MTT	thiazolyl blue tetrazolium Bromide
MW	molecular weight
NEB	negative energy balance
NEFA	non-esterified fatty acids
OD	optical density
p.p.	post partum
p38-MAPK	mitogen-activated protein kinase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered salt solution
PCR	polymerase chain reaction
PP	primiparous
PPARα	peroxisom-proliferator- activated receptor α
PVDF	polyvinylidene diflouride
rec.	recombinant
ROS	reactive oxygen species
RT	room temperature
sc. AT	subcutaneous adipose tissue
SCC	somatic cell count
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SP	seminal plasma
TBS	tris buffered saline
TBST	tris buffered saline – tween
TED	tris-carboxymethylethylenediamine
TNF	tumor-necrosis factor
vc. AT	visceral adipose tissue

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CHAPTER I: General introduction

1. Introduction

Cattle are used for milk and meat production and contribute greatly to the human food supply. The use of high-yielding dairy breeds such as Holstein-Friesians has resulted in an increase in milk production during the past 50 years. Dairy cows have been selected to produce ~55 kg milk per day, which is three times the milk yield of dairy cows 30 to 40 years ago (Breves, 2007). However, milk production exposes animals to a variety of stressors. Dairy cattle are susceptible to an increased incidence and severity of diseases during the periparturient period, which is the time from late pregnancy to early lactation (Sordillo et al., 2009). After parturition, milk production increases rapidly and body reserves are mobilized to cover the energy lost with milk. Consequently, the cow drifts into a negative energy balance (NEB). Energy balance is defined as the ratio between the energy consumed and the energy required for maintenance, growth, pregnancy and lactation (Grummer, 2007). Metabolic adaptations to NEB include increased hepatic gluconeogenesis and the increased mobilization of fatty acids from adipose tissue (AT) and amino acids from muscle (Bell, 1995). Many other bodily functions are related to energy balance, e.g. the postpartum ovarian activity depends on the energy balance of the cow (Beam and Butler, 1999). Over-nutrition has been found to reduce placental-fetal blood flow and thus fetal growth in sheep (Wallace et al., 2002). Promoting optimal nutrition will therefore not only ensure optimal fetal development, but will also reduce the risk of chronic diseases in later life (Wu et al., 2004). Generally, AT not only serves as an energy store, it is also an endocrine organ; AT secretes hormones named adipokines. This term is restricted to proteins secreted from adipocytes, and excludes signals that are released only by other cell types (such as macrophages) in the AT (Trayhurn and Wood, 2004). Adipokines can act locally within the AT, but they can also reach distant organs through the blood circulation. In their target organs, adipokines can exert a wide range of biological actions. They are involved in lipid metabolism, insulin sensitivity, the alternative complement system, vascular homeostasis, blood pressure regulation and angiogenesis. In addition, there is a growing list of adipokines that are involved in inflammation and the acute-phase response (Trayhurn and Wood, 2004). One of the most abundant adipokines in the circulation is adiponectin (AdipoQ). Adiponectin in negatively correlated with body fat content and is known to be a key regulator of insulin sensitivity and tissue inflammation (Whitehead et al., 2006).

Adiponectin has been studied intensively in humans and rodents, whereas research about bovine AdipoQ has been impeded by the lack of valid, species-specific assays. Adiponectin occurs in a number of different molecular weight (MW) forms that are assumed to be of different biological importance. Therefore, this thesis is focused on establishing Western blot methods to characterize AdipoQ MW forms in different body fluids at different physiological stages of cattle.

2. Literature review

2.1. The adipokine adiponectin

Adiponectin is one of the most abundant adipokines found in the circulation, with concentrations of around 0.01% of total serum proteins. Adiponectin was first described in the 1990s in mouse and human plasma (Scherer et al., 1995; Nakano et al., 1996). Sato et al. (2001) first isolated bovine AdipoQ. It is primarily secreted by adipocytes (Arita et al., 1999) and plays important roles in the regulation of glucose and lipid metabolism (Waki et al., 2003). Contrary to other adipokines produced by AT, e.g. Leptin and Visfatin, AdipoQ is inversely correlated with body mass and insulin resistance (Arita et al., 1999). High concentrations of AdipoQ lead to decreased gluconeogenesis and reduced intracellular triglyceride content in the liver, whereas glucose uptake in skeletal muscle is stimulated by AdipoQ (Waki et al., 2003). Furthermore, AdipoQ exerts immunological functions; it regulates the expression of several pro- and anti-inflammatory cytokines. Its main anti-inflammatory function is potentially related to its capacity to suppress the synthesis of tumor-necrosis factor α (TNFα) and interferon-γ (IFNγ). Moreover, it is able to induce anti-inflammatory cytokines such as interleukin-10 (IL-10) (Tilg and Moschen, 2006).

2.1.1. Adiponectin structure and expression

Bovine AdipoQ is a polypeptide of 240 amino acids which structurally belongs to the complement factor 1q family (Sato et al., 2001). The amino acid sequence of bovine AdipoQ shows 92% homology with human AdipoQ and 82% homology with murine AdipoQ (Sato et al., 2001). Generally, the primary amino acid sequences of AdipoQ are highly conserved across species; sharing over 80% identity among all of the species cloned so far (Wang et al., 2008). Adiponectin consists of different structural domains (Fig. 1); it has a secretory signal

sequence at the N-terminal part (amino acids 1–17), a variable region, which is the species-specific region (Waki et al., 2003), a collagenous region (amino acids 45–111) and a globular domain (amino acids 112– 240) (Sato et al., 2001).

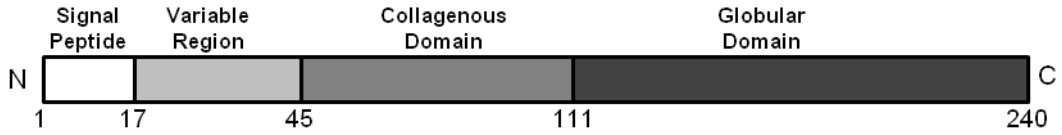

Fig.1: Structural domains of bovine adiponectin. Numbers indicate the first amino acid of the corresponding domain (modified according to Wang et al., 2008)

Adiponectin is modified extensively at the post-translational level during secretion from adipocytes. The amino acid residues of AdipoQ with known post-translational modifications are highly conserved among different species (Wang et al., 2008). Adiponectin is synthesized as a single 28 kDa monomer which undergoes multimerization to form multimers of different molecular weight (MW) forms prior to secretion (Fig. 2) (Waki et al., 2003). Low molecular weight (LMW) AdipoQ is composed of three monomers (combining to form a trimer) resulting in a size of 67 kDa. A hexamer formed by two trimers represents the middle molecular weight (MMW) form of AdipoQ with a size of 136 kDa. The high molecular weight (HMW) multimers of AdipoQ are comprised of 12 to 18 monomers and reaches a MW of more than 300 kDa (Waki et al., 2003).

In humans and mice, the HMW AdipoQ is the most abundant form circulating in the serum (Pajvani et al., 2003; Tsao et al., 2003). All modifications in MW are due to post-translational modifications like hydroxylation and glycosylation (Wang et al., 2004). Thereby, the conserved proline and lysine residues in the collagenous domain are hydroxylated and afterwards glycosylated. The characteristic oligomeric isoforms are created by disulfide bonds at the cysteine in the variable region (Waki et al., 2003).

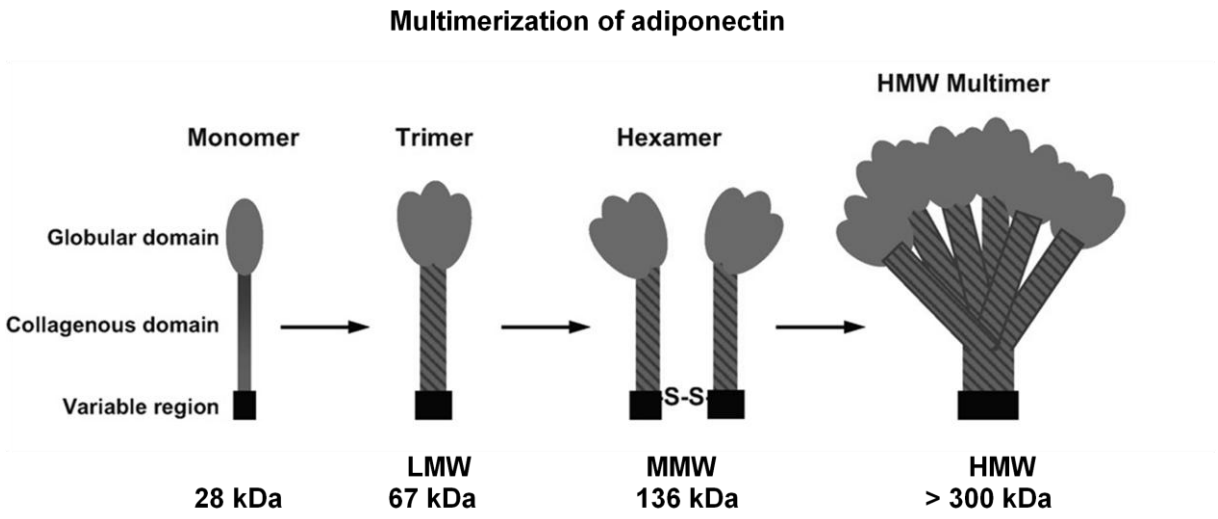

Fig. 2: Multimerization of adiponectin. LMW = low molecular weight, MMW = middle molecular weight, HMW = high molecular weight, and S-S = disulfide bonds (modified from Simpson and Whithead 2010)

2.1.2. Adiponectin receptors and signaling

Adiponectin has three receptors that are found in different tissues: adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) (Yamauchi et al., 2003) and the cell surface protein T-cadherin (Hug, 2004). T-cadherin is believed to be one of the AdipoQ binding proteins because of its missing intracellular domain and its lack of expression in hepatocytes (Kadowaki et al., 2006). AdipoR1 and AdipoR2 belong to the seven transmembrane receptor family; they have an intracellular amino terminus and an extracellular carboxyl terminus. AdipoQ signaling is mediated by several transcription factors and intracellular receptors (Fig. 3). Free AdipoQ binds to the N-terminal extracellular domain, whereas the intracellular C terminal domain binds to APPL1 (an adaptor protein containing a pleckstrin homology domain, a phosphotyrosine binding domain and a leucine zipper motif) (Mao et al., 2006; Cheng et al., 2007; Thundyil et al., 2012). APPL1 acts as a link between the receptors and their signaling molecules. The signaling molecules activated by AdipoQ include adenosine monophosphate-activated protein kinase (AMPK), mitogen-activated protein kinase (p38-MAPK), and peroxisome proliferator activated receptor α (PPARα) (Thundyil et al., 2012). Adiponectin signaling is down regulated by AMPK. Activation of AMPK by AdipoQ leads to decreased gluconeogenesis in the liver (Kadowaki and Yamauchi, 2005). The activation of PPARα by AdipoQ increases fatty acid oxidation in liver and muscle, and p38-MAPK activation by AdipoQ causes glucose uptake in muscle (Kadowaki and Yamauchi, 2005).

AdipoR1 mainly acts via the AMPK pathway, whereas AdipoR2 acts through the PPARα pathway (Yamauchi et al., 2007). Furthermore, it was shown that the AdipoQ receptors bind different MW forms of AdipoQ: AdipoR1 has a strong affinity for globular and full length AdipoQ, while AdipoR2 has an intermediate affinity for full-length and globular adiponectin (Whitehead et al., 2006).

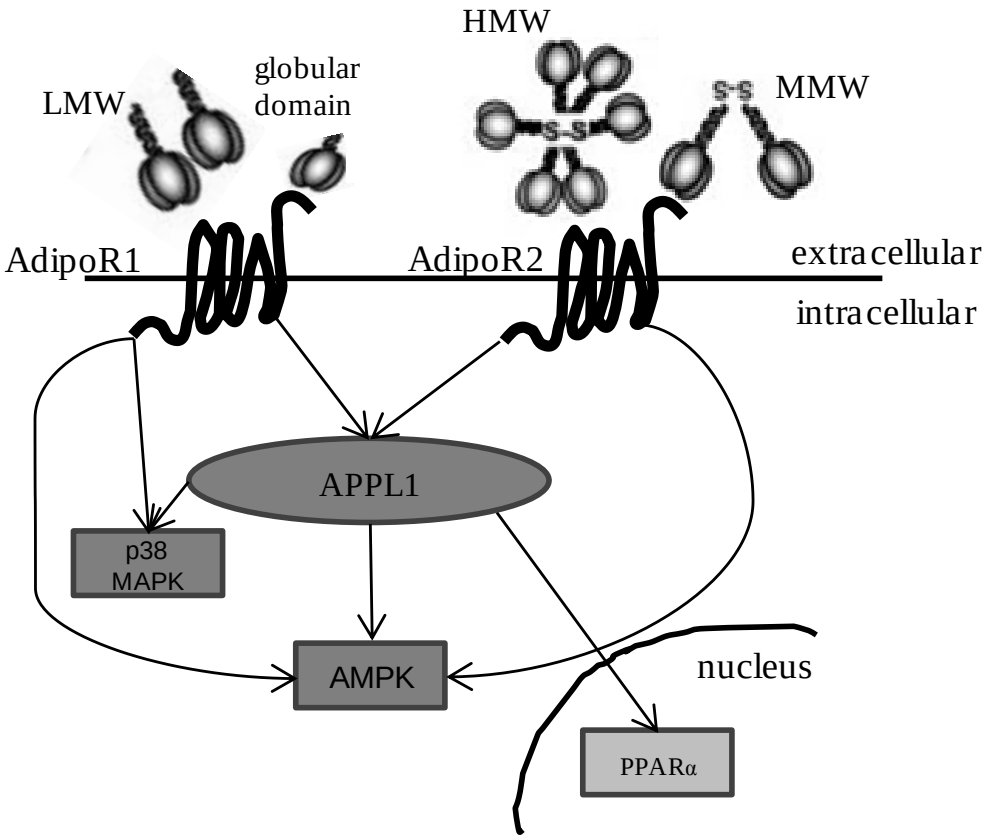

Fig. 3: Adiponectin receptors and signaling. AdipoR1/R2 = adiponectin receptor 1 and 2, APPL1 = adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif, AMPK = adenosine monophosphate-activated protein kinase, PPARα = peroxisome proliferator activated receptor α, p38-MAPK = mitogen-activated protein kinase, LMW = low molecular weight, MMW = middle molecular weight, HMW = high molecular weight, and S-S = disulfide bonds (modified from Thundyil et al., 2012)

2.2. Importance of adiponectin in cattle

2.2.1. The transition period

The transition period starts three weeks ante partum (a.p.) and ends three weeks post partum (p.p.). It is the most critical phase of the lactation cycle for dairy cows (Grummer, 1995). This time determines the profitability of dairy cows, because the ability to reach maximal production efficiency can be impeded by health problems, nutrient deficiency or poor management (Drackley, 1999). During this periparturient period, the energy requirements of dairy cows increase; to cover the output of energy via milk, cows start to mobilize body fat and muscle tissue. The rapidly increasing demands of glucose, amino acids and fatty acids for milk production cannot be sufficiently compensated for by feed intake alone. The reduction of dry matter intake around parturition is caused by alterations related to metabolic, physical, behavioral and hormonal changes (Allen et al., 2005). Consequently, the cows enter a state of negative energy balance (NEB). To direct glucose towards the mammary gland, the insulin sensitivity of peripheral tissues, e.g. muscle and adipose tissue (AT), is reduced (Bell, 1995). With low insulin concentrations in the serum and reduced insulin sensitivity, lipolysis in AT starts, which leads to an increase in serum concentrations of non-esterified fatty acids (NEFA) (Drackley et al., 2005). The uptake of NEFA into the liver during excessive lipolysis results in a risk of the development of fatty liver and possible negative effects on neutrophil function (Scalia et al., 2006). The circulating concentrations of β-hydroxybutyrate (BHB) are associated with the oxidation of fatty acids in the liver: BHB increases with the incomplete oxidation of fatty acids in the liver (Leblanc, 2010). Elevated BHB and NEFA concentrations lead to a higher incidence of ketosis and may also result in infectious diseases in like mastitis and metritis due to compromised immune function (Drackley, 1999).

The role of AdipoQ in dairy cows is of special interest in the transition period because of its insulin-sensitizing effect (Whitehead et al., 2006). In dairy cows, the abundance of AdipoR1 and AdipoR2 mRNA in subcutaneous adipose tissue was significantly different when comparing a.p. and p.p. (Lemor et al., 2009). Adiponectin mRNA abundance increased in visceral (v.s.) AT with increasing days in milk (Saremi et al., 2014), but no information about the course of AdipoQ protein concentrations and the MW forms in the transition period was available until now.

2.2.2. Immune status of cows during the transition period

Periparturient inflammatory diseases, like mastitis or puerperal fever, occur within the first two weeks after calving (Ohtsuka et al., 2004). Complex relationships between immune function and metabolic status exist. Impaired leukocyte function contributes to the susceptibility for infectious diseases in the periparturient period (Harp et al., 1991; Detilleux et al., 1995). The concentration of neutrophils, lymphocytes and monocytes varies from eight weeks a.p. to eight weeks p.p. With the exception of monocytes, all blood immune cells are increased one to two weeks prior to parturition, while these cell populations are lowest at parturition and in the first week p.p. (Meglia et al., 2005). Lymphocyte number decreases until parturition, mainly due to reduced lymphocyte proliferation (Kehrli et al., 1989). Furthermore, bovine blood lymphocytes are less responsive to mitogen stimulation, e.g. concanavalin A (ConA) (Nonneke et al., 2003). The proliferative activity of ConA-stimulated bovine peripheral blood mononuclear cells (PMBC), lymphocytes, monocytes and macrophages is reduced p.p. in comparison to the proliferative ability of PBMCs in mid-lactating cows (Shafer-Weaver and Sordillo, 1997).

The major function of neutrophils is the elimination of infiltrated bacteria, mainly by phagocytosis. The functionality of neutrophils seems to be associated with NEB in the dairy cow (LeBlanc, 2012). Neutrophil phagocytosis and oxidative burst were increased during *in vitro* experiments in which they were incubated with early p.p. serum, reflecting the physiologically high NEFA concentrations found in serum at parturition (Ster et al., 2012). Scalia et al. (2006) observed no effects for moderate concentrations of NEFA, but reported an increase in phagocytosis-induced oxidative burst at high concentrations (> 1mM). Additionally, the PBMC proliferation from mid-lactating cows is known to be negatively affected by incubation with the serum of early lactating cows, which naturally contains higher NEFA concentrations compared to those seen mid-lactation. The impaired immune function might be more related to the serum composition at the beginning of lactation than to a defect of the immune cells (Ster et al., 2012). The increase of plasma NEFA is likely to exert negative effects on lymphocyte functions in cows (Lacetera et al., 2004). With increasing NEFA concentrations in the culture medium, PBMC reduce proliferation and the secretion of cytokines, e.g. IFNγ. Furthermore, the secretion of immune globulin M (IgM), which is an indicator of acute inflammation, is reduced (Lacetera et al., 2004).

The potential effects of AdipoQ on immune cell function are mainly studied in human cell cultures.

Expression of AdipoQ and its receptors was shown in human bone marrow mononuclear cells (Crawford et al., 2010) and AdipoR1 was found to be expressed in human T-lymphocytes (Takahashi et al., 2010). Furthermore, low expression of the AdipoQ protein in lymphocytes has also been observed (Crawford et al., 2010). Adiponectin provides the ability to decrease the secretion of TNFα and IFNγ in human T-lymphocytes (Takahashi et al., 2010). The induced secretion of TNFα and IL-6 of porcine macrophages by lipopolysaccharides (LPS), part of the cell membrane of gram-negative bacteria, is reduced by pre-incubation of these cells with AdipoQ. This suggests that the anti-inflammatory actions of AdipoQ include suppression of pro-inflammatory cytokines, e.g. IL-6, and the induction of anti-inflammatory ones, e.g. IL-10 (Wulster-Radcliffe et al., 2004).

2.2.1.2. Adiponectin in reproduction

Reproductive success is closely linked to energy balance, whilst metabolic dysregulation is linked with reproductive abnormalities (Schneider, 2004). The length of the p.p. anovulatory period is strongly associated with NEB through a decrease of luteinizing hormone (LH) pulse frequency and low levels of blood glucose and insulin, which collectively limit estrogen production by dominant follicles (Butler, 2003). Lower fertility in dairy cows is related to NEB as a result of the effects that are exerted early in lactation and later during the breeding period (Butler, 2003). Energy homeostasis is regulated by AdipoQ through the modulation of glucose and fatty acid metabolism in peripheral tissues (Dridi and Taouis, 2009). Adiponectin and its receptors are expressed in several tissues besides adipose and liver tissue (Table 1). The expression of AdipoQ mRNA was found in several tissues related to reproduction: rat pituitary gland (Rodriguez- Pacheco et al., 2007), chicken testis (Ocon-Grove et al., 2008), bull spermatozoa (Kasimanikman et al., 2013), human placenta (Caminos et al., 2005), and ovary (Chabrolle et al., 2007). Additionally AdipoQ mRNA was demonstrated in human lymphocytes (Crawford et al., 2010). The expression of AdipoR1 and R2 in the human pituitary gland suggests a feedback of the gonadotropic axis by AdipoQ (Psilopanagioti et al., 2009). In addition, AdipoQ is involved in the regulation of pituitary hormone secretion: it reduces the GnRH-stimulated LH secretion through the increased phosphorylation of AMPK (Lu et al., 2008). The influence of AdipoQ on LH secretion in the pituitary gland was confirmed in cultured rat pituitary cells (Rodriguez-Pacheco et al., 2007).

Recently, the mRNA and protein expression of AdipoR1 and R2 was shown in the porcine pituitary gland. Kiezun et al. (2013) showed that the expression of AdipoQ receptor mRNA and protein expression is affected by the stage of the estrus cycle in sows. The presence of both ligand and receptors in the porcine pituitary may suggest an auto-/paracrine role for AdipoQ in the regulation of the function of this gland (Kiezun et al., 2013). In particular, the expression of AdipoR2 differs throughout the estrus cycle; the highest expression of AdipoR2 was found during the luteal phase, which might be related to increasing steroid hormone concentrations (Kiezun et al., 2013).

Adiponectin is discussed as a potential marker for fertility. The expression of AdipoQ and its receptor mRNA and protein was shown in the bovine female reproductive system. The physiological status of the ovary has significant effects on the natural expression patterns of AdipoQ and its receptors in follicular and luteal cells of the bovine ovary (Tabandeh et al., 2010). The expression of AdipoQ mRNA in bovine granulosa cells of follicles (11-22 mm) is positively correlated with estradiol concentration in follicular fluid (Tabandeh et al., 2010). With increasing follicular size, the expression of AdipoQ and AdipoQ receptor mRNA increases in bovine follicles, especially in cumulus and granulosa cells (Tabandeh et al., 2010). Adiponectin further decreases insulin-induced steroidogenesis in cultured bovine granulosa cells (Maillard et al., 2010). A positive correlation between serum and follicular fluid (FF) AdipoQ concentrations has been shown in women. Moreover, the AdipoQ concentration in FF was shown to be about five times lower than in serum (Bersinger et al., 2006). Beside these differences in AdipoQ concentrations, the isoforms of AdipoQ also differ between serum and FF in women: in FF, the LMW (trimer) AdipoQ is the most abundant MW form, whereas in serum, the HMW is the major AdipoQ MW form (Bersinger et al., 2010).

Table 1: Expression of Adiponectin (AdipoQ) and its receptors in several tissues

Tissue/ cells	**Species**	**AdipoQ**		**AdipoR1**		**AdipoR2**		**Reference**
		mRNA	Protein	mRNA	Protein	mRNA	Protein	
Pituitary gland	human			+		+		Psilopanagioti et al., 2009
	rat	+		+		+		Rodriguez-Pacheco et al., 2007
	porcine		+	+	+	+	+	Lu et al., 2008
Follicular cells	bovine	+	+	+	+	+	+	Tabandeh et al., 2010 Maillard et al., 2010
Luteal cells	bovine	+		+		+		Tabandeh et al., 2010
Ovary	bovine	+	+	+	+	+	+	Tabandeh et al., 2010 Maillard et al., 2010
Testis	rat	+	+					Caminos et al., 2008
	chicken	+	+					Ocon-Gove et al., 2008
Fetal tissue: skin, skeletal muscle, gut	human	+	+					Corbetta et al., 2005
Amniotic membrane	human	+	+					Corbetta et al., 2005
Placenta	human		+					Corbetta et al., 2005
Mammary gland	bovine	+		+		+		Saremi et al., 2014
T-lymphocyte	human			+				Takahashi et al., 2010

2.2.4. Physiological regulation of milk production

Milk production is mainly controlled by the hormones prolactin and growth hormone (GH). These hormones are essential for the transition from a proliferating to a lactating mammary gland (Svennersten-Sjaunja and Olsson, 2005). Growth hormone is the dominating hormone in ruminants (Flint and Knight, 1997); it increases the blood flow in mammary glands and has blood glucose-elevating effects. Prolactin increases the intestinal uptake of calcium and the uptake of fatty acids into the udder, which are necessary for milk fat synthesis (Svennersten-Sjaunja and Olsson 2005). The elements that are necessary for milk synthesis are provided by the circulation; therefore, the blood flow through the mammary gland increases with decreasing days until parturition. In sheep, blood flow in the mammary gland increases at parturition, whilst milk yield is correlated with mammary blood flow until peak lactation is reached (Nielsen et al., 1990).

The main components of milk secreted by the mammary gland are fat, protein, and minerals. The composition of milk is dependent on the stage of lactation, breed, parity and the energy status of the cow (Grieve et al., 1986). In relation to milk yield, the components of milk vary during the lactation period. While lactose decreases during the course of lactation, fat and protein concentrations in milk increase after an initial decline.

Adiponectin, as the insulin-sensitizing hormone with glucose-lowering effects (Yamauchi et al., 2003), is present in human milk. The concentrations range from 4 to 88 ng/mL (Newburg et al., 2010), depending on maternal nutritional status and diet during pregnancy. The concentration of AdipoQ in milk is lower compared than that reported in the serum. In human milk, AdipoQ was suggested to play a role in the early growth and development of breast-fed infants (Woo et al., 2009). Maternal AdipoQ milk concentrations are negatively correlated with the infant's weight at birth and in the first two months. The MW form of AdipoQ found in human milk is predominantly HMW (Woo et al., 2009). Different results concerning milk AdipoQ concentrations throughout the course of lactation are available. Martin et al. (2006) showed a decrease of up to 6% in human milk AdipoQ concentrations with each month of lactation. They further mentioned that AdipoQ in milk increases in the period after parturition with increasing maternal post-pregnancy body mass index (Martin et al., 2006). Differing results concerning the AdipoQ concentrations in human milk over the lactation period have been described. Decreasing milk AdipoQ concentrations in the first 6 months of lactation were shown by Woo et al. (2009).

In an additional human study, significantly higher milk AdipoQ concentrations in the first week of lactation were observed compared to mature milk in the third month of lactation (Ley et al., 2012). In contrast to the above-mentioned results, the lowest AdipoQ concentrations (13.3 ± 0.6 ng/mL) were reported in human colostrum (day 1-3 p.p.), followed by an increase in milk AdipoQ concentration up to 180 days of lactation (Ozarda et al., 2012).

2.2.5. Ontogenesis of adiponectin secretion

The findings in human studies have led to the assumption that AdipoQ might be involved in fetal development. One indication for this is that the expression of AdipoQ mRNA and protein in fetal skin, skeletal muscle, gut and amniotic membrane, is at apparently lower levels than those expressed in adult white AT (Corbetta et al., 2005). Adiponectin in amniotic fluid is supposed to be of fetal origin, because of the lack of correlation with maternal serum AdipoQ concentrations (Baviera et al., 2007). Moreover, AdipoQ seems to be associated with early neonatal growth in humans. It was shown that cord blood AdipoQ concentrations in term-born neonates were higher than serum concentrations in adults (Arita et al., 1999; Inami et al., 2007). Positive correlations for AdipoQ cord blood concentrations with birth length and birth weight were described. Adiponectin might exert its influence on fetal growth, which is controlled by insulin and IGFs, by acting as an insulin sensitizer (Inami et al., 2007). When present in human cord blood, AdipoQ is associated with a slower weight gain in the first six months of life and not related to adiposity in three years of age (Mantzoros et al., 2009).

At birth and at one month of age, no differences in serum AdipoQ concentrations were found between male and female infants (Inami et al., 2007; Kamoda et al., 2004). In contrast, a sexual dimorphism of serum AdipoQ concentrations was described in adult humans and rodents; AdipoQ concentrations are lower in male than in female rats, mice and humans (Combs et al., 2003; Pajvani et al., 2003; Arita et al., 1999). With increasing age, the serum concentrations of AdipoQ in rats increased independently of sex (Combs et al., 2002 and 2003). An increase in serum AdipoQ concentrations with age (18-78 years) was also observed in humans (Schautz et al., 2012).

Besides the possible influences of AdipoQ on growing individuals, it also appears to be involved in reproductive processes. Adiponectin in female reproduction is described in 2.2.1.2.

The contribution of AdipoQ to male reproduction was shown in human and rodent studies. Adiponectin mRNA and protein expression was found in rat and chicken testis (Table 1) (Caminos et al., 2008; Pfaehler et al., 2012; Ocon-Gove et al., 2008). Particularly in the testosterone-secreting leydig cells of rat and chicken testis, AdipoQ mRNA and protein were localized (Caminos et al., 2008; Ocon-Gove et al., 2008).

AdipoQ concentrations in rodents are influenced by androgens; serum testosterone concentrations are negatively correlated with serum AdipoQ concentrations in humans and rats (Page et al., 2005; Nishizawa et al., 2002). Moreover, orchidectomy or ovarectomy in rats leads to increased AdipoQ serum concentrations. Additionally, testosterone treatment of castrated male rats resulted in decreasing serum AdipoQ concentrations (Yarrow et al., 2012). Also, recombinant AdipoQ significantly inhibited basal and human choriogonadotropin-stimulated testosterone secretion in rat testicular tissue *in vitro* (Caminos et al., 2008).

3. Objectives

Adiponectin is known to have various effects on energy metabolism and immunity. Dairy cows undergo several metabolic changes during the transition from late pregnancy to early lactation. These metabolic changes might lead to immunocompromised situations and the mobilization of body reserves in early lactating dairy cows. For that reason, the management of transition cows has been shown to be necessary to underpin production and profitability on dairy farms.

A few studies have already provided results on AdipoQ in cattle, but they were mainly based on mRNA data. Less is known about the distribution of AdipoQ MW forms in several tissues and fluids; some of the above-mentioned studies have described the patterns of AdipoQ in humans and rodents but these results cannot be generalized for cattle. Due to the lack of valid bovine-specific assays, research on bovine AdipoQ has been impeded until now. We aimed to establish methods to quantify the amount of AdipoQ protein in serum and other body fluids. First, a semi-quantitative Western blot was established and validated to estimate AdipoQ concentrations in the serum and milk of dairy cows during the first weeks of lactation. An in-house quantitative bovine-specific AdipoQ ELISA has been developed, which was established in a cooperative thesis by S.P. Singh (2014); this was applied to several samples which have also been used in the present thesis. Additional information about the origin and functionality of AdipoQ in various physiological states of cattle may be provided by a qualitative description of AdipoQ MW patterns. Therefore, a non-reducing, non-denaturing (semi-native) Western blot protocol was developed to classify the different AdipoQ MW forms at different stages of lactation and in several body fluids and tissues of cattle.

Additionally, recombinant bovine AdipoQ was expressed and produced in *Escherichia coli* to test the functional ability of recombinant AdipoQ on lymphocyte proliferation *in vitro*.

CHAPTER II: Methodological developments and first pilot studies

Western Blot (WB) is a commonly used method for determination of the presence of proteins by specific antibodies. The proteins are separated according to their size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS is used as a detergent, which is applied to the protein samples to linearize proteins and to dissociate them from each other. SDS binds non-covalently to proteins to impart a negative charge so that all proteins are directed to the anode whilst running in the gel (GE Healthcare handbook). Afterwards, the separated proteins are electro-transferred onto a membrane. The detection of the targeted protein is achieved by incubating the membrane with a specific primary antibody. A conjugated, e.g. horseradish peroxidase (HRP) labeled, secondary antibody is then used to bind the protein-primary antibody complex. The chemiluminescent substrate reacts with the enzyme (HRP) and the substrate produces light, which can be detected by exposing the blot to a digital imaging system using a camera, or by exposing the blot to an X-ray film (Fig. 4).

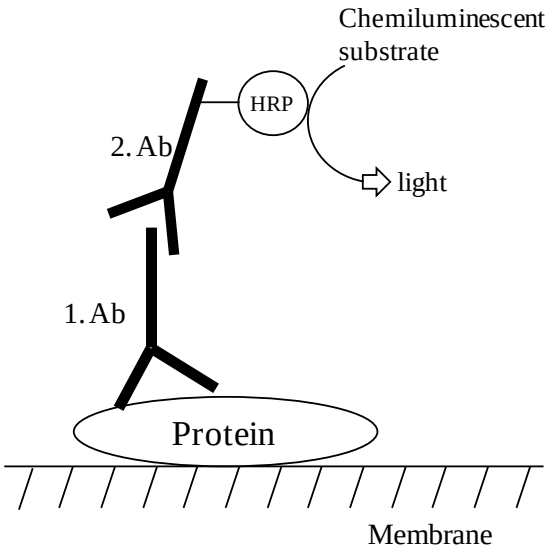

Fig. 4: Detection of the target protein by Western blot with the use of a primary antibody (1. Ab) and a secondary antibody (2. Ab) labeled with horseradish peroxidase (HRP) (modified from www.cellsignal.com/pdf/7071.pdf)

Western blot is used as a qualitative method to test for the existence of the protein of interest in a sample which contains various different proteins. It can also be used as a semi-quantitative method; for that purpose, the amount of protein loaded per lane has to be identical for all samples on a given gel. After detection, the relative concentration of the target protein is estimated by comparison between the signal intensities of the sample and a control sample of known concentration.

Adiponectin is a protein which is secreted in different MW forms, as described before (Chapter I_ 2.1.1.). In human serum, three different complexes of AdipoQ were detected; LMW (trimer) (~ 67 kDa), MMW (hexamer) (136 kDa) and HMW (multimer) (> 300 kDa) (Waki et al., 2003). By reducing and denaturing the samples before using them for a Western blot, it is possible to convert the AdipoQ multimers to monomers (Fig. 5).

In the present thesis, a semi-quantitative Western blot protocol was established for bovine AdipoQ. For that purpose, we estimated the total amount of AdipoQ in a sample by comparing the intensity of the samples with the intensity of a pooled sample. Total AdipoQ was determined by reducing and denaturing the samples before using them in SDS-PAGE. The treatment generates monomers and dimers (Fig. 5B, lane 4) which were detected on the membrane, the intensities of both bands were added and the relative amount of total AdipoQ was calculated.

Besides the semi-quantitative Western blot, we established a semi-native Western blot protocol to detect the MW patterns of AdipoQ in several bovine body fluids and tissues. For this, the samples were neither reduced nor denatured before applying them to the SDS-PAGE, so that AdipoQ was visible in its native MW forms (Fig 5B, lane 1). With this method, differences in the AdipoQ MW distribution that are dependent on the physiological stage of the researched cattle can be identified.

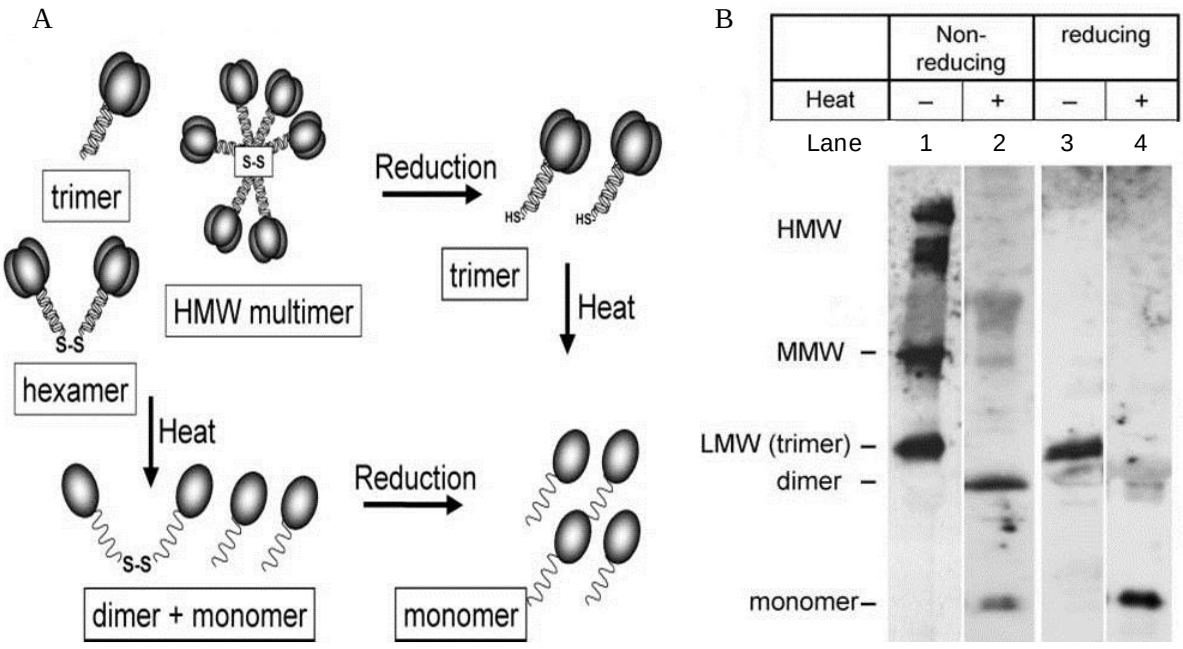

Fig. 5: (A) Structure of AdipoQ MW forms and their variation by with reducing or denaturing treatment. (B) Exemplary Western blot of human serum AdipoQ treated by heat or reduction or both (modified from Waki et al., 2003)

The following chapter will explain the different Western blot protocols, their validation and first pilot studies. All buffers and solutions used for both Western blot protocols are provided in detail in appendix A.

1. Development, validation and first application of a semi-quantitative Western blot for bovine adiponectin

1.1. General set-up

After optimization, which included identifying the optimal blotting time, gel concentration and antibody dilution, we established a protocol as described below.

First, the samples were diluted with PBS and mixed with sample buffer. To estimate the amount of total adiponectin, it is necessary to convert the AdipoQ multimers to dimers and monomers. Therefore, the disulfide bonds of the multimers were reduced by dithiothreitol (DTT, 200 mM) and denatured by boiling (5 min, 95°C). After centrifugation for 5 min at 10,000 × *g* at 4°C, 5 µl of each sample were applied to SDS-PAGE. For this, each of the samples, the reference standard and the MW marker (Precision Plus Protein WesternC Standards, BIO-RAD, Munich, Germany) were loaded in duplicate onto a 5.6% stacking gel and electrophoresed through a 12% tris-glycine polyacrylamide gel (Carl Roth, Karlsruhe, Germany). The gel running was performed using an SE260 mighty small II deluxe mini vertical electrophoresis unit (Hoefer, Inc., Holliston, MA, USA) according to the method of Laemmli (1970). Two gels were run simultaneously at 150 V until the dye front had proceeded almost to the end of the gel.

After electrophoresis, the gels were equilibrated for 10 min in Towbin blot buffer. Before use, the polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Munich, Germany) had to be activated for 10 sec in 100% methanol, washed in distilled water and then stored in blot buffer until further use. Six filter papers (0.7 mm, Macherey-Nagel, Düren, Germany) were equilibrated in blotting buffer before packing the blot sandwich. The first three filter papers were placed on the anode and rolled out carefully to exclude air bubbles in the blotting sandwich. Afterwards, the membrane with the gel on top was placed on the filter papers. To complete the sandwich, the remaining three filter papers were placed on top of the pack and rolled out carefully to eliminate all air bubbles. Transfer of the proteins was performed with a Trans-Blot Turbo transfer unit (Bio-Rad Laboratories, Munich, Germany) over a period of 25 min at 25 V and maximally 1.0 A.

After completing the blotting procedure, the membranes had to be blocked to prevent interactions between the membrane and the antibody used for the detection of AdipoQ since the membranes are established to bind proteins as well as antibodies. The open binding sites of the membrane were blocked by incubating with Tris-buffered saline containing Tween 20 (TBST) for 60 min at RT. The membranes were exposed to the primary antibody, rab14 R-3, in a 1: 4000 (0.25 µg/ mL) dilution for 1 h at RT and then washed (4 x 5 min) with TBST. Subsequently, the membranes were treated with the secondary goat-anti-rabbit antibody, conjugated with horseradish peroxidase (HRP) (1:50,000; SouthernBiotech, Birmingham, AL, USA). After washing again (4 x 5 min), the immune complex was detected with the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Amersham, UK, RPN 2135). For this, the membranes were covered by a mixture of equal volumes (500 µl) of each ECL detection reagent 1 and 2 in a final volume of 20 µL/cm^2 and incubated for 5 min in the dark without agitation. Afterwards, the detection reagent was drained off and the membranes were sealed in plastic and placed in the imaging system. Imaging was performed with a VersaDoc MP4000 imaging system (Bio-Rad). The membranes were detected for 1 min and the band intensities were densitometrically analyzed via the ImageLab software (Bio-Rad).

1.2. Validation of the semi-quantitative Western blot protocol

Following the denaturation and reduction of serum samples, the two expected bands were detected: the AdipoQ monomer (~ 28 kDa) and the dimer (~ 56 kDa) (Fig. 6B). Linearity of the semi-quantitative Western blot was proven by estimating the coefficient of regression (R^2) between the totaled band intensities per lane and against the dilution factor (Fig. 6A).

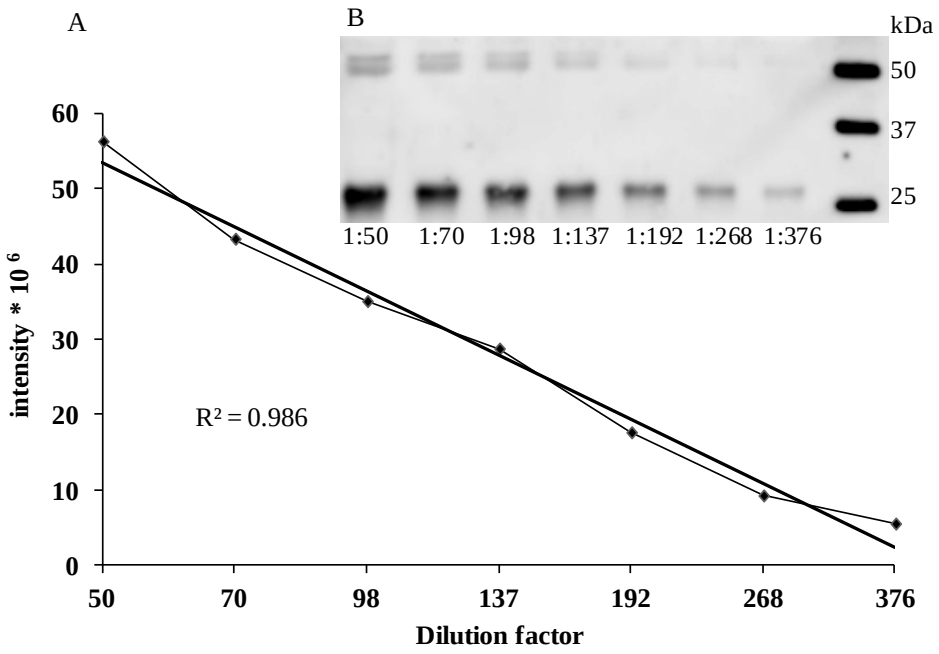

Fig. 6: Linearity of diluted serum samples in semi-quantitative Western blot. (A) Total intensity of both bands, estimated by ImageLab, was plotted against the dilution factor. (B) Exemplary Western blot of individual diluted serum samples. Adiponectin monomer and dimer estimated by comparison to the molecular weight marker

Reducing and denaturing resulted in the pattern for adiponectin which had already been shown in human studies (Waki et al., 2003; Nakano et al., 2006). Adiponectin circulates in higher MW forms in human plasma. To calculate the amount of total adiponectin, it was necessary to convert the multimers into monomers. The treatment used here (95°C, 5 min and 200 nM DTT) was obviously not sufficient to destroy all disulfide bonds. The incomplete conversion of dimers was also shown in humans (Nakano et al., 2006). The reproducibility of the semi-quantitative estimation of bovine AdipoQ is shown by an intra-assay variation of 15.7% and an inter-assay variation of 46.5%. In addition, repeated freezing and thawing of serum samples did not influence the estimated amount of AdipoQ.

1.3. Application of the semi-quantitative Western blot protocol to characterize the concentration of adiponectin during lactation in serum and milk of dairy cows

1.3.1. Animals and blood and milk sampling

Serum samples were collected by jugular vein puncture from six multiparous Holstein dairy cows that were repeatedly sampled on days –21, –14, –7, 1, 7, 14, 21, 35, 49, 70, 105, 140, 182, 189, 196, 210, 224, 238, and 252 relative to parturition. Samples were then centrifuged (3,000 × *g*, 20 min, 4°C) and serum samples were stored at -20°C until further use.

Milk samples (15 mL total composite milking) were taken during the morning milking procedure from three multiparous Holstein dairy cows from day 5 p.p. until day 21 p.p., three times a week.

1.3.2. Sample preparation and Western blot

Serum and samples, as well as the corresponding reference standard (a pool of dairy cow serum or milk samples), were diluted 1: 150 with 1× PBS, and mixed 1: 4 with sample buffer before loading onto the gel. The downstream protocol is described in the general set up (1.1).

1.3.3. Statistical analyses

The mean intensities of the bands corresponding to the monomers (28 kDa) and dimers (56 kDa) were totaled for each lane, and then the means of the duplicates from samples and standards were calculated. Results are presented as the ratio between the sample and standard (serum or milk pool) intensities. If the variation between sample duplicates was higher than 20%, the sample was repeated. In cases where there was variation of over 20% in the pooled sample, the whole gel was repeated.
For evaluation of the time course of adiponectin serum concentrations in cows during late pregnancy and lactation, the mixed model with repeated measurement was applied with the use of IBM SPSS Statistics 19.0 (IBM, Ehningen, Germany).

1.3.4. Results and discussion

The first application of the semi-quantitative estimation of AdipoQ was performed to characterize the AdipoQ concentration in serum over an entire lactation period. Serum AdipoQ concentrations decreased towards parturition with a nadir at day seven a.p. (Fig. 7). After parturition, the concentrations increased again until a plateau was reached for the next 252 days in lactation. Serum concentrations of AdipoQ were significantly lower a.p. than p.p.; in addition, the estimated AdipoQ concentrations of semi-quantitative Western blot correlated ($r = 0.626$, $P < 0.001$, $n = 114$) with the concentrations measured by ELISA.

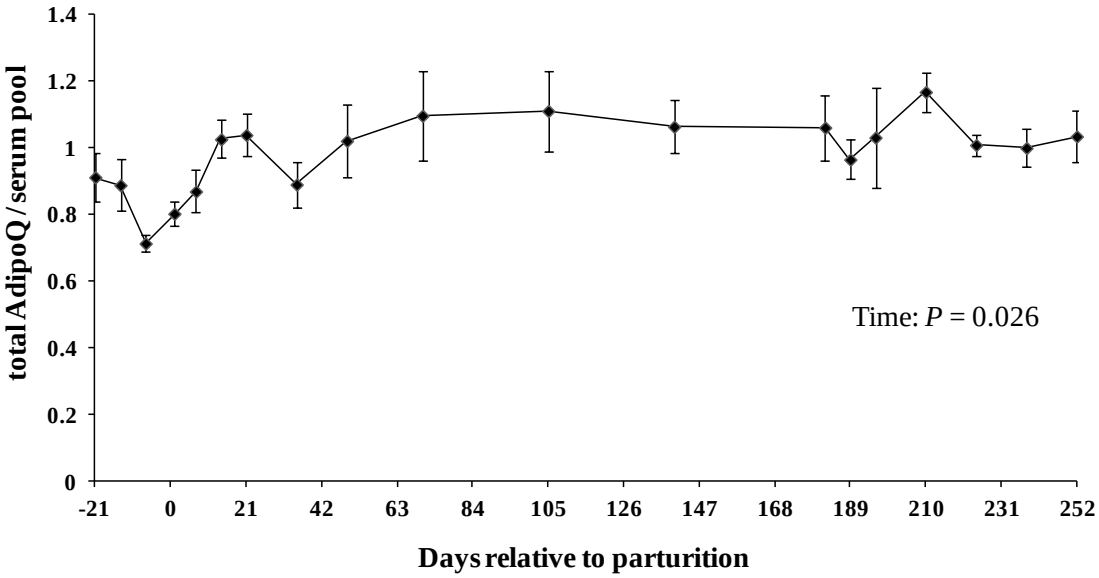

Fig. 7: Relative adiponectin serum concentrations (means ± SEM) normalized to a standard serum pool in the time from late pregnancy until 252 days of lactation in 6 Holstein cows

In human maternal blood, the AdipoQ concentrations decline during late pregnancy and reach the lowest concentrations after parturition (Fuglsang et al., 2010; Corbetta et al., 2005). Decreasing AdipoQ concentrations towards lactation might be related to the common decrease in insulin sensitivity. Towards lactation, the glucose requirement of the bovine mammary gland increases, which leads to a decrease in the availability of glucose in adipose tissue (Bell and Baumann, 1997). Supporting these findings, Giesy et al. (2012) suggested that the decreasing AdipoQ concentrations around parturition were a physiological mechanism to improve gluconeogenesis and glucose supply to the mammary gland for milk production. The hormonal changes associated with parturition are probably related to the changes in circulating AdipoQ because AdipoQ expression and secretion are reportedly suppressed by prolactin and growth hormone *in vitro* (Nilsson et al., 2005).

In view of the body fat mobilization occurring during the first weeks of lactation and the known inverse relationship of AdipoQ and body fat content, an increase rather than a decrease of serum AdipoQ concentrations was expected. The physiological priority of lactation might be the reason why the relationship between body fat and AdipoQ is uncoupled in favor of maintaining insulin resistance.

Samples of skimmed milk could be used in Western blot without any methodical adjustment. Milk samples taken from 3 cows at day 5 to day 21 in the lactation period were used to estimate the milk AdipoQ time course. The AdipoQ concentration in milk slightly decreased

throughout the first three weeks. The concentration at day 5 in lactation was 2- fold higher compared to the concentration at day 8 and 11 in lactation (Fig. 8).

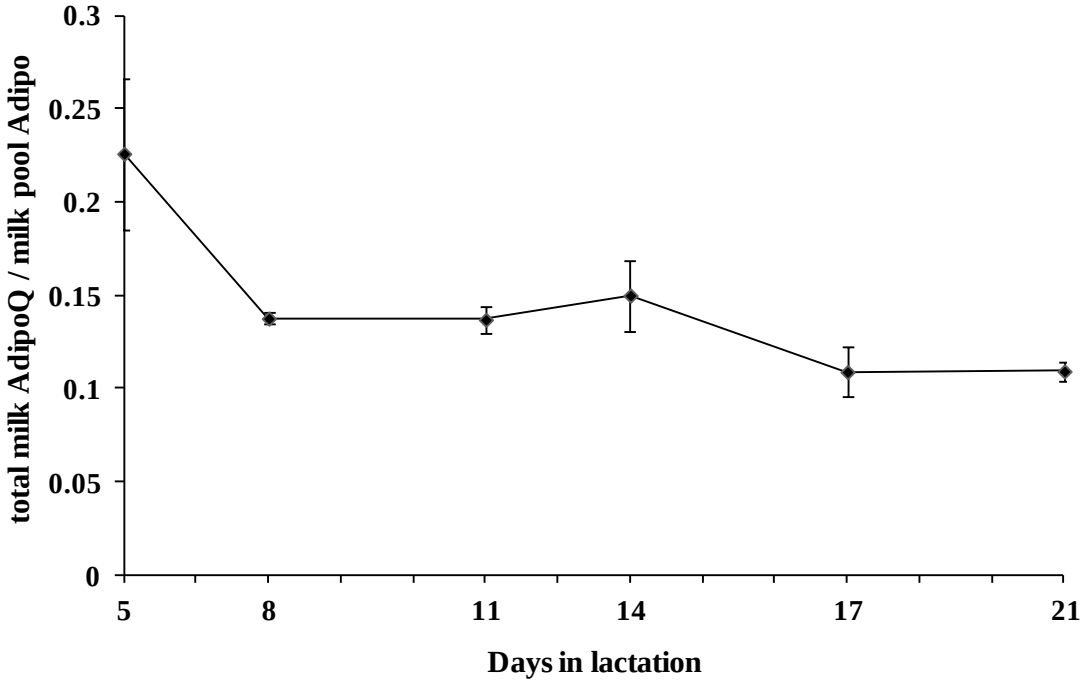

Fig. 8: Relative adiponectin concentrations (means ± SEM) in skimmed milk of three cows from day 5 until day 21 in lactation

Adiponectin concentration in milk appears to be the opposite of the above-described serum concentrations in the first weeks of lactation. Milk AdipoQ concentrations in women decline throughout lactation (6 months) (Martin et al., 2006; Woo et al., 2009). Since we focused on the first three weeks of lactation, no comparison to human concentrations within the subsequent months can be drawn. In line with our findings, the AdipoQ concentration in human early milk was higher compared to mature milk (Ley et al., 2012; Martin et al., 2006). However, contradictory results have been published; e.g. Ozarda et al. (2012) found increasing AdipoQ concentration in breast milk over time during lactation.

Decreasing milk AdipoQ concentration could be explained by the rapid changes in milk composition during the first weeks of lactation (Blum and Hammon, 2000).

2. Development, validation and first application of the qualitative (semi-native) Western blot protocol for bovine adiponectin

2.1. General set up of the semi-native Western blot

The AdipoQ concentrations of all samples used in the semi-native Western blot were previously estimated using the in-house ELISA (Mielenz et al., 2013). To compare the MW distribution of AdipoQ, all samples were analyzed under non-reducing, non-denaturing conditions. For this, samples were diluted with PBS to an equal amount of AdipoQ per lane and mixed with sample buffer. Before loading on the SDS gel, all samples were centrifuged for 5 min at 10,000 × *g* at 4°C; 10 µl of each sample were loaded onto a 5.6% stacking gel and electrophoresed through an 8% tris-glycine polyacrylamide gel (Roth, Karlsruhe, Germany). The samples were run under reducing conditions because of the SDS contained in the running buffer. The gel running was performed using an SE260 mighty small II deluxe mini vertical electrophoresis unit (Hoefer, Inc., Holliston, MA, USA) according to the Laemmli method (Laemmli, 1970). Two gels were run simultaneously starting with 50 V for 15 min and continuing at 150 V until the dye front had proceeded to the end of the gel.

Proteins separated by SDS PAGE were transferred onto a PVDF membrane by the use of tank blotting with the Criterion Blotter System (Bio-Rad Laboratories, Munich, Germany). For transferring proteins of higher molecular weight, the methanol concentration in Towbin buffer was reduced to 10%. The blot sandwich assembly started by placing one foam pad on the black wire electrode (cathode). Three filter papers soaked in blot buffer were placed above. Before the gel was put on top of the filters, they were rolled carefully to eliminate all air bubbles in the blotting sandwich. The gel on top of the filter papers was covered with the membrane and three remaining filter papers. Again, the sandwich was rolled out to exclude any air bubbles trapped in the sandwich. Then, the second foam pad was placed on top of the blotting sandwich and the electrode was closed. The tank blot apparatus was filled with 1.3 L of Towbin blot buffer, an ice block and a magnetic stirrer. Before starting the transfer, the wire electrodes and the blot sandwiches were placed in the tank. The transfer was performed at 100 V for 60 min with stirring. The blotted membranes were detected as described previously for the general set-up of the semi-quantitative Western blot (1.1). In addition to the Versa Doc detection, the membranes were detected using CL-XPosure films (Thermo Scientific, Germany) to produce sharp and intensive bands.

2.2. Validation of the semi-native Western blot protocol

To evaluate the sensitivity of the polyclonal antibody, a serum dilution series with known AdipoQ concentrations (ELISA) was used. With increasing dilution, the intensity of the bands decreased, with the HMW band disappearing and the MMW band becoming fainter (Fig. 9).

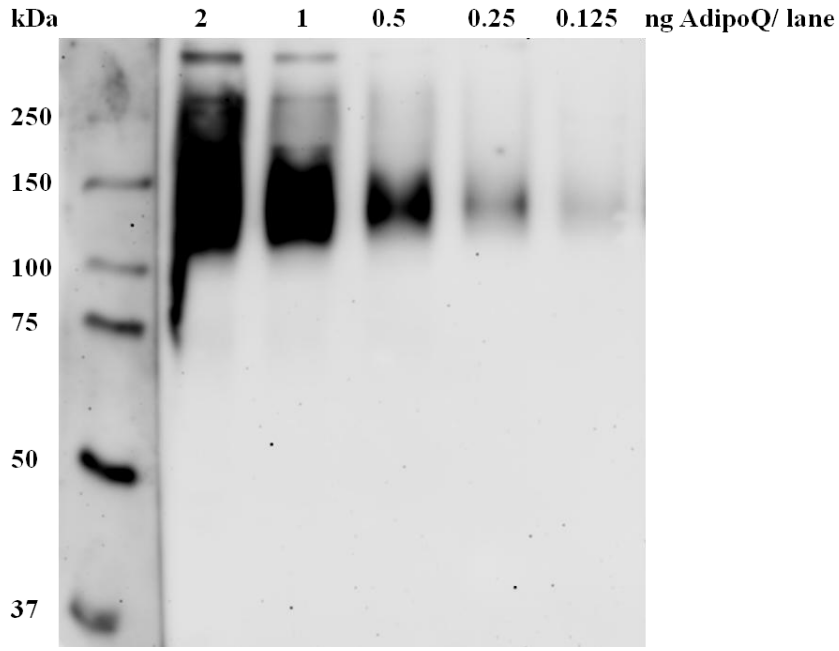

Fig. 9: Dilution series of serum samples analyzed by semi-native Western blot using 8% SDS-PAGE

The serum dilution series clearly indicated that with decreasing AdipoQ concentrations, the HMW band was no longer visible. These findings led to the decision to use an AdipoQ serum concentration of 1 ng/lane for further analyses in this study.

Different reducing and denaturing treatments of serum samples led to the expected differences in MW forms of AdipoQ (Fig. 10). The lane containing the untreated sample showed two bands: one HMW (> 250 kDa) and one intensive MMW (> 130 kDa) band. Heat treatment alone was not able to break the disulfide bonds, meaning that the bands showed the same pattern as the untreated sample, by using denaturation and reduction monomers as well as residual dimers of expected sizes of ~ 28 kDa and ~ 56 kDa were found.

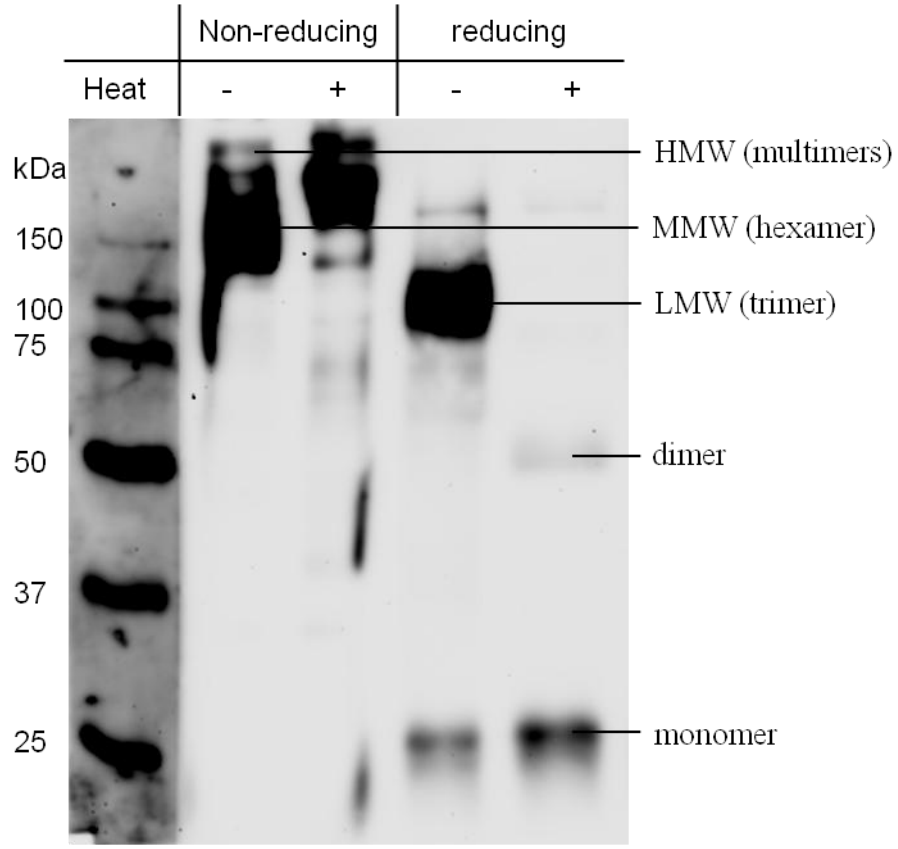

Fig. 10: Characteristic changes of adiponectin MW forms with the use of denaturing and reducing conditions for Western blot analysis. Bovine serum (dilution 1:200) subjected to the indicated conditions; 10% SDS-PAGE with or without heat denaturation (95°C, 5 min), and with or without reducing conditions (200 mM dithiothreitol)

With reducing and denaturation treatment, we observed different MW forms of AdipoQ, as already reported for humans and mice (Waki et al., 2003). It seems that the percentage of MMW (hexamer) AdipoQ in bovine serum is higher than all other MW forms.

Our bovine specific polyclonal antibody detected the same AdipoQ pattern as that observed previously with a bovine specific monoclonal antibody (Raffelspieper et al., 2012). In this study, the MMW AdipoQ band was also the most prominent, which supports the hypothesis that the MMW form of AdipoQ might be the most abundant in cattle.

The distribution of AdipoQ complexes observed by gel filtration chromatography differed between human and mouse serum; a lower level of trimeric AdipoQ was observed in mice (Schraw et al., 2008). Furthermore, male subjects (humans and mice) had an equal distribution of AdipoQ, whereas females showed a decline in the proportion of complexes; HWM is the most abundant followed by LMW (trimer) and finally the trimer (Schraw et al., 2008).

Pajvani et al. (2003) showed that the portion of HMW to LMW (trimer) was different between male and female mice. Female mice had a higher ratio of HMW to LMW, whereas the male mice had more LMW and less HMW. The amount of LMW AdipoQ in these mice was not found to be different between sexes (Pajvani et al., 2003).

The complex distribution of AdipoQ is further influenced by body fat content (lean vs. obese) in humans; obesity and insulin resistance lead to lower levels of the HMW AdipoQ in serum (Schraw et al., 2008). In addition, Kovacova et al. (2009) estimated the secreted MW complexes of AdipoQ in subcutaneous (sc. AT) and viceral adipose tissue (vc. AT) in comparison to the distribution in serum of obese and non-obese humans. Obesity affected the secretion of AdipoQ MW forms in AT in a fat depot-dependent manner; it was found to be associated with the lower secretion of total adiponectin in sc. AT but not in vc. AT, whereas the relative proportion of HMW was higher in vc. AT than in sc. AT in non-obese subjects and did not differ between the two depots in obese subjects (Kovacova et al., 2009).

These findings lead to the hypothesis that bovine AdipoQ MW might also differ concerning adipose tissue distribution in the transition period. Therefore, we applied the newly established AdipoQ MW blot protocol to the serum of cows from different lactation stages and numbers.

2.3. First application of the semi-native Western blot protocol to characterize the molecular weight distribution of adiponectin during lactation in serum and milk of dairy cows

2.3.1. Animals and serum sampling

Serum samples from eight lactating Holstein cows were analyzed to show the different MW forms of AdipoQ. Samples were selected from two multiparous and two primiparous cows at day 1 and day 105 p.p., respectively.

Milk samples (15 mL total composite milking) were taken during the morning milking procedure from three multiparous Holstein cows from day 5 p.p. until day 21 p.p., thrice a week. The same milk samples as those described in 1.3.1 were used.

2.3.2. Sample preparation and Western blot procedure

Serum samples were used at concentrations of 1 ng AdipoQ/lane and skimmed milk samples at 0.4 ng AdipoQ/lane. The following Western blot protocol describes the general set-up of the semi-native Western blot (2.1).

2.3.3. Results and discussion

The serum concentrations estimated by ELISA at day 1 p.p. were significantly lower than on day 105 p.p. (Singh et al., 2014b). Different serum concentrations might be related to different secretion patterns of AdipoQ. Therefore, we aimed to investigate possible differences in MW patterns of AdipoQ between early and late lactation and concerning to the cows parity.
The MW patterns of AdipoQ did not vary between the lactation stage and the parity of the cows (Fig. 11); they mirrored the regular bovine serum pattern observed before, with a strong MMW band and a faint HMW band.

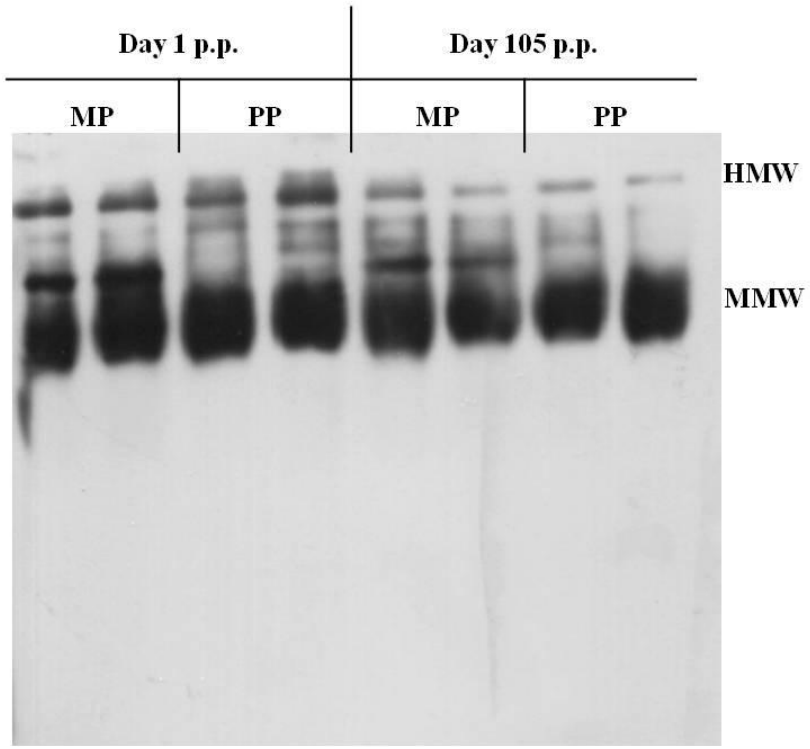

Fig. 11: Exemplary semi-native Western blot of the adiponectin molecular weight forms of dairy cows at different stages of lactation (Day 1 and 105 post partum (p.p.)) and different parity; multiparous (MP) and primiparous (PP). High molecular weight (HMW) and low molecular weight (LMW)

Neither day 1 vs. day 105 of lactation nor parity affected the MW distribution of AdipoQ. The available data indicate that parity can influence the pattern of changes in blood hormones and metabolites, since PP animals are still growing and nutrients are directed to fetal and maternal growth and in milk production.

In general, by the second lactation, maternal growth is almost completed and milk production has increased considerably (Coffey et al., 2006). In contrast, pregnancy in MP cows is established during lactation and milking is usually finished about 6 wks. prior to the expected calving. The metabolic status is different in animals at the start of second and subsequent lactations (Wathes et al., 2007) and thus results in different concentrations among blood metabolites of PP and MP animals. Thus the physiological reasons for variations in AdipoQ serum concentrations might not be related to the MW secretion patterns of AdipoQ.

To define the MW pattern of AdipoQ in milk during early lactation, milk samples from three multiparous Holstein dairy cows were used.

The AdipoQ MW patterns in mature milk were apparently not different to those seen before in the serum (Fig. 12).

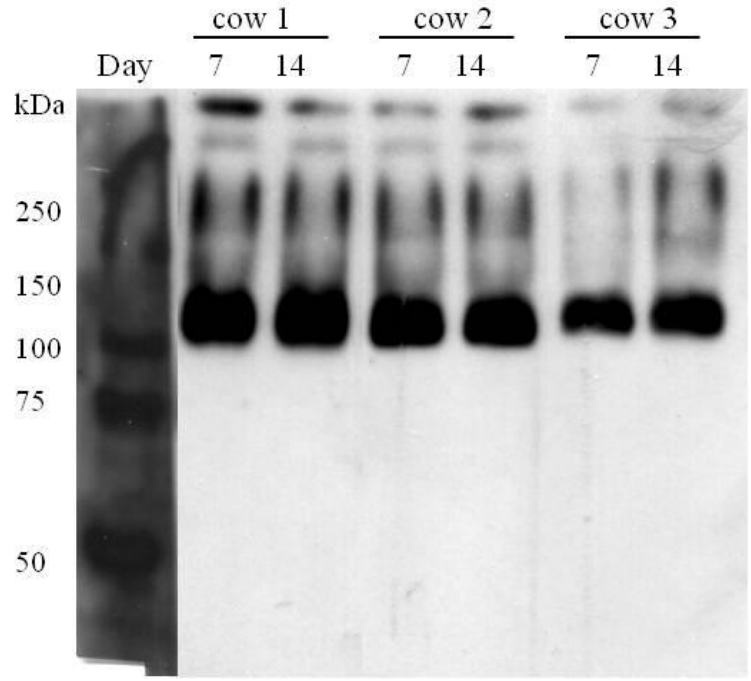

Fig. 12: Exemplary semi-native Western blot of adiponectin in milk samples from three cows on different days in lactation

The given similarity of serum and milk AdipoQ MW patterns might indicate that milk AdipoQ arises from circulation into the mammary gland. However, the MW distribution in human milk only showed the HMW band of AdipoQ, potentially due to methodical differences. In the latter study, native PAGE was used to identify the AdipoQ MW pattern, which is not comparable to the semi-native Western blot method used here (Woo et al., 2009).

Further studies of AdipoQ MW patterns in different physiological stages and in several fluids and tissues are described in Chapter III.

CHAPTER III: Molecular weight patterns of adiponectin in different body fluids and tissues estimated by semi-native Western blot

1. Adiponectin molecular weight pattern in milk and serum samples from experimentally-induced mastitis

A common mastitis pathogen is the bacterium *Escherichia coli (E. coli)*; its negative effects on the mammary gland are mediated by lipopolysaccharides (LPS), i.e. parts of the cell membrane of gram negative bacteria. Invading pathogens induce an immune response in the mammary gland by activating the innate immune system, mainly neutrophils. During an immune response in the mammary gland, the blood-milk barrier is negatively affected and becomes leaky (Burton and Erskine, 2003). This causes not only an increase in somatic cells in milk but also further increases the concentration of blood-borne factors in milk, e.g. BHB (Lehmann et al., 2013). The aim of this experiment was to examine the effect of experimentally-induced mastitis on mature milk and blood AdipoQ MW patterns.

The experimental design was described in detail previously (Lehmann et al., 2013). Briefly, healthy mid-lactation MP Holstein cows (n = 7) were used. All udder quarters were free of clinical symptoms of mastitis with SCC < 150,000 cells/mL. After the morning milking, 200 µg *E. coli* LPS (serotype O26:B6; Sigma-Aldrich, Saint Louis, MO, USA) diluted in 10 mL 0.9% sterile saline solution were infused into the left front quarter. Milk samples (~20 mL) from the experimental quarter were collected immediately before and 8 h after the LPS infusion. Samples were skimmed (4,000 × *g*, 15 min, 4°C) and frozen at -20°C until analysis. Blood samples were collected before and 90 min after LPS infusion, and thereafter at hourly intervals until 8.5 h after LPS application; plasma samples were obtained by centrifugation (3,000 × *g*, 20 min) and stored at -20°C until analysis.

For Western blot analysis, serum and milk samples taken before and after LPS challenge were used to characterize the potential effects of mastitis on AdipoQ MW patterns. Milk and serum samples were used at a concentration of 0.7 ng AdipoQ/lane.

The distribution of AdipoQ MW complexes did not vary before and after LPS challenge, and neither in serum samples nor in milk samples (Fig. 13).

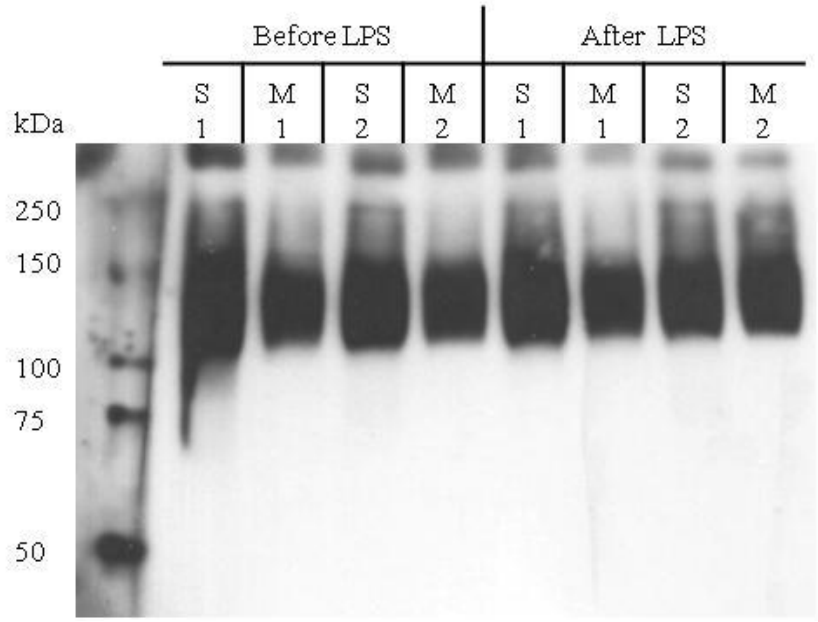

Fig. 13: Exemplary semi-native Western blot of milk (M) and serum (S) samples from cows with experimentally-induced mastitis. Samples of two (1, 2) Holstein cows before and after treatment with *E. coli* lipopolysaccharide (LPS)

Although the AdipoQ concentrations in milk measured by ELISA increased after the LPS challenge (Singh et al., 2014a), the MW pattern of AdipoQ did not show any differences. Acute mammary infections are known to alter milk composition, even though these components might not have any immunological importance, like an increase in milk lactate and BHB (Lehmann et al., 2013). It seems that experimentally-induced mastitis with LPS did not change the MW secretion pattern of AdipoQ neither in milk nor in serum, which additionally supports the hypothesis that milk AdipoQ arises from the circulation.

2. Adiponectin molecular weight patterns in visceral and subcutaneous adipose tissue depots and mammary gland

The following section investigates the MW patterns of AdipoQ in different AT depots at different stages of lactation. Adipose tissue samples from three primiparous lactating Holstein cows from the control group of a feeding experiment described elsewhere (von Soosten et al., 2011) were used. The lactating dairy cows were slaughtered on either day 1, 42 or 105 of lactation. Adipose tissue from three sc. depots (tailhead, withers and sternum) and three vc. depots (omental, mesenteric and retroperitoneal), as well as the mammary gland, was sampled. Tissues were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

After thawing the tissue samples on ice, they were homogenized in two volumes of homogenization buffer using the Precellys® 24 homogenizer (Peqlab Biotechnologies GmbH, Erlangen, Germany). Homogenates were centrifuged twice at 14,000 × *g* for 10 min at 4°C to separate the fat layer. The supernatants (without fat and tissue debris) were collected and stored at -20°C until analysis.

Analysis of AdipoQ MW pattern was performed by semi-native Western blot, using 0.5 ng AdipoQ/lane, as described in Chapter II (1.2.3 and 1.2.4).

The characterization of AdipoQ MW patterns was done using different AT depots, homogenates from vc. (mesenterial, omental, retroperitoneal) and sc. (withers, sternum, tail head) AT and from the mammary gland. As a reference, the corresponding serum samples were also loaded on the gel.

Different AT and mammary gland homogenates showed no apparent differences in the MW pattern of AdipoQ. In all fat tissues a HMW and an MMW band was detectable (Fig. 14)

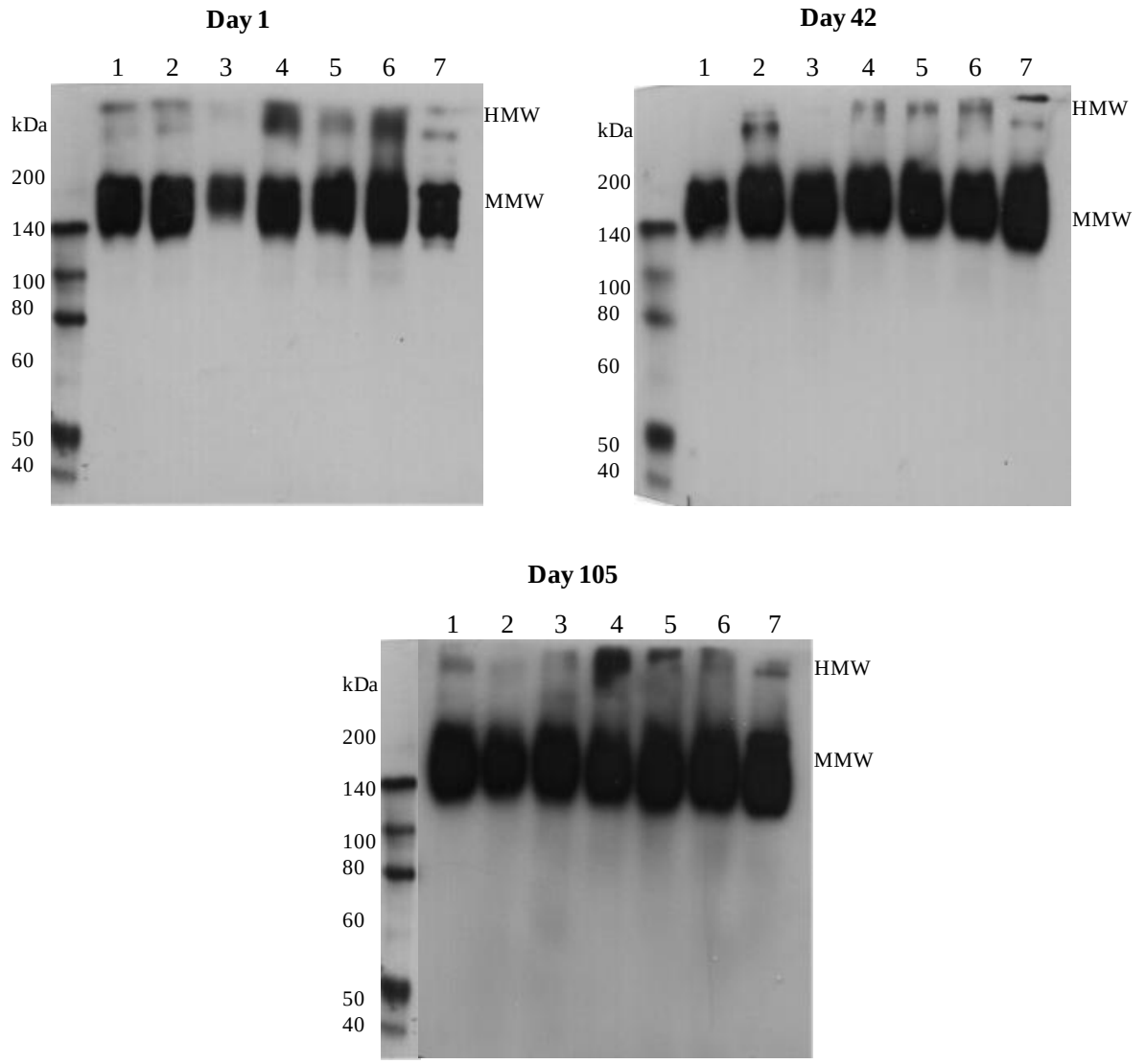

Fig.14: Exemplary semi-native Western blots of AdipoQ molecular weight pattern in adipose tissue (AT) extracts and serum on day 1, day 42 and day 105 of lactation. *Lane 1*: mesenterial AT extract; *Lane 2*: omental AT extract; *Lane 3*: retroperitoneal AT extract; *Lane 4*: wither AT extract; *Lane 5*: tail-head AT extract; *Lane 6*: sternum AT extract; *Lane 7*: serum

There was also no effect of lactation stage on the MW patterns of AdipoQ in vc. and sc. AT. Furthermore, the variation in AdipoQ concentrations in these tissue homogenates was not mirrored by the AdipoQ MW pattern. No variation of serum AdipoQ MW patterns in the transition period was seen in a bovine study (Giesy et al., 2012) and within our results (see chapter II). Human studies detected differences in the secretion amount and pattern in sc. AT and vc. AT. Subcutaneous AT plays a greater role in the release of total and HMW AdipoQ (Meyer et al., 2013).

The ratio of secreted HMW versus total AdipoQ in vc. AT and sc. AT explants was higher in non-obese subjects compared to obese humans (Kovacova et al., 2012); however, our cows were not obese, and actually had a body condition score of 3 (Pappritz et al., 2011). Therefore, changes in AdipoQ concentrations might not be reflected by changes in the MW patterns of AdipoQ in bovine AT.

In view of AT samples obtained through biopsies or collected after exsanguination at slaughter, the samples might contain residual blood. Singh et al. (2014b) estimated that the amount of residual blood in the tissue samples with the use of the transferrin concentration in the tissue preparations and in serum was able to correct for blood-derived AdipoQ. However, the estimated blood content in AT samples was negligible (>1%) in tissue samples derived after exsanguination and was low (5% of mg total protein) in biopsy samples (Singh et al., 2014b).

The expression of AdipoQ in mammary glands was detected by Western blot (Fig. 15); the patterns were similar to those found for serum and milk previously (Chapter II, 2.3.3.).

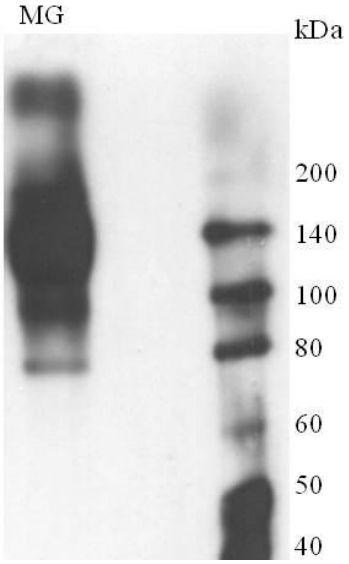

Fig. 15: Adiponectin molecular weight pattern in the bovine mammary gland (MG) estimated by semi-native Western blot

The expression of AdipoQ mRNA in the bovine mammary gland was previously reported (Saremi et al., 2014; Ohtani et al., 2011). Only a marginal expression of AdipoQ mRNA in mammary gland was found (Saremi et al., 2014; Giesy et al., 2012), which is not in support of parenchyma being a meaningful source of AdipoQ. However, the expression of AdipoQ receptors in the mammary gland indicates a physiological relevance of AdipoQ in milk production. Adiponectin receptor mRNA abundance in the mammary gland increased during the course of lactation (Saremi et al., 2014).

The similarity of mammary gland AdipoQ MW patterns to serum AdipoQ MW may indicate that AdipoQ from serum is mainly present in the mammary gland homogenate. This may further explain the low expression of AdipoQ mRNA in the mammary gland; the low signal might contribute to stromal adipose cells, which might be part of the homogenate. The low AdipoQ mRNA expression in mammary glands during lactation was accounted for by the decreasing number of stromal adipose cells in lactating mammary glands (Ohtani et al., 2011).

3. Adiponectin molecular weight patterns in cerebrospinal fluid (CSF)

Cerebrospinal fluid (CSF) is a liquid which surrounds the central nervous system. The hypothalamus and the brainstem are parts of the brain and are involved in the regulation of feed intake. Cerebrospinal fluid contains blood metabolites and hormones whose concentrations depend on the functioning of the blood brain barrier. Consequently, CSF hormone and metabolite concentrations differ from those in the blood. As already mentioned in the literature review of Chapter I, dairy cows tend to develop a negative energy balance in the transition period, which is caused by reduced feed intake and increasing milk production. One part of the regulation of feed intake is played by adipokines, which circulate in blood and CSF at different concentrations. Leptin decreases in serum and CSF after calving, whereas ghrelin, the counterpart of leptin, did not vary in concentration (Laeger et al., 2013). As AdipoQ is one of the most abundant adipokines in circulation and as this study showed a decrease in serum concentration around parturition, we were interested in studying the pattern of AdipoQ in the CSF of dairy cows around parturition.

Cerebrospinal fluid samples were collected from ten German Holstein dairy cows at day −20, −10, +1, +10, +20, and +40 relative to parturition and were provided by Dr. B. Kuhla, Leibnitz Institute of Farm Animal Biology (FBN) Dummerstorf. The details of the experiment are described elsewhere (Laeger at el., 2013). Briefly, the CSF from spinal cord was obtained by lumbar puncture and blood was withdrawn from the jugular vein before morning feeding. Blood samples were centrifuged at 1,573 × *g* for 20 min and CSF samples at 4,566 × *g* for 5 min (each at 4°C). Plasma and CSF aliquots were frozen in liquid nitrogen and stored at −80°C until analysis (Laeger at el., 2013). The semi-native Western blot procedure was conducted according to the method described in Chapter II (1.2.3 and 1.2.4). The concentration of 0.7 ng AdipoQ/lane was used for serum samples and CSF was used at a concentration of 0.3 ng AdipoQ/lane.

Generally, the AdipoQ concentration in serum was about 80-fold higher compared to that in CSF. At Day -20 and +40 relative to parturition, serum AdipoQ concentrations were 1.2-fold higher compared to day +1 and +10, as confirmed by ELISA measurement. Cerebrospinal fluid and serum samples of individual days in the transition period showed no apparent differences in the MW pattern of AdipoQ (Fig. 16).

The MMW band was the most prominent one in all cases, whereas the HMW tended to be faint in all samples, independent of whether serum or CSF was analyzed.

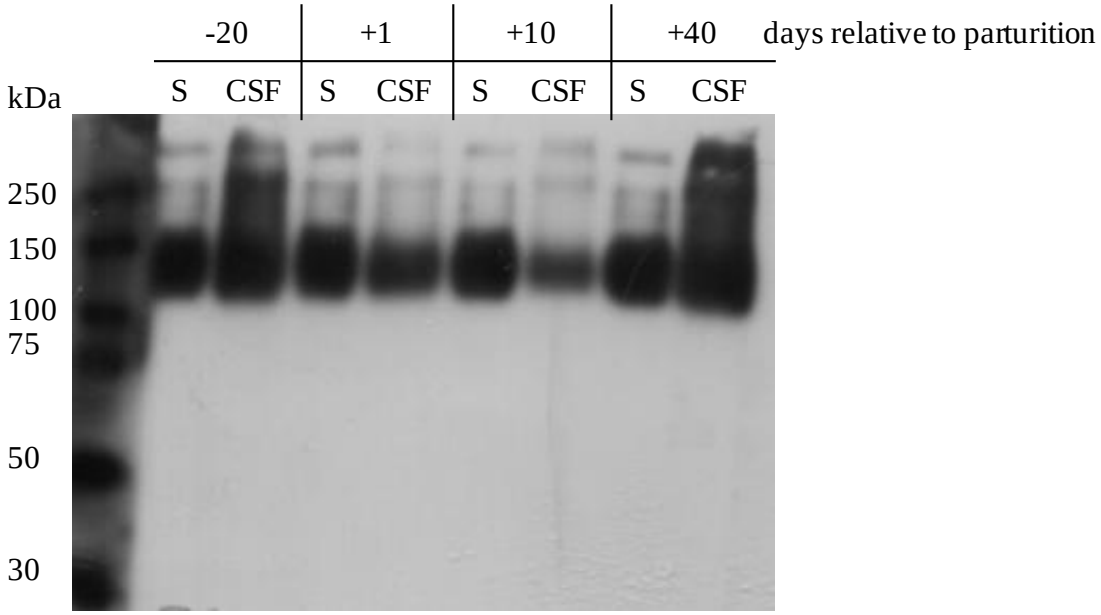

Fig. 16: Exemplary semi-native Western blot of AdipoQ molecular weight patterns in cerebrospinal fluid (CSF) and corresponding serum samples (S) from one cow

In contrast to AdipoQ MW patterns found herein for bovine CSF, human CSF did not show any HMW band, neither by Western blot (Neumeier et al., 2007) nor by gel filtration chromatography and subsequent semi-quantitative Western blotting (Kusminski et al., 2007). Our results also showed no differences between serum and CSF MW patterns of AdipoQ. The most abundant band of AdipoQ was the MMW in both serum and in CSF. Furthermore, the MW pattern in serum or CSF did not differ at different stages of the transition period. Although there were variations in serum AdipoQ concentrations in the transition period, which have already been described in Chapter II (2.1.1) the MW pattern of AdipoQ did not vary (Chapter II 2.2.2). The transfer of AdipoQ through the blood brain barrier, which had already been shown in human studies (Kusminski et al., 2007; Neumeier et al., 2007), might explain the similarity of MW patterns found in serum and CSF.

4. Adiponectin in *Bovidae* other than *Bos taurus*

As Yak, Bison and Water buffalo are also used for milk and meat production it is of interest to compare the distribution of AdipoQ MW patterns to *Bos taurus*. In Europe, buffalos are mainly used for milk production. A characteristic of buffalo milk is the high percentage of both protein and fat in the milk, although the average milk yield per lactation is much lower compared to dairy cattle (Rosati and Van Vleck, 2002) which might protect buffalos from suffering metabolic changes during the transition period.
Our aim was to investigate AdipoQ patterns in cattle other than *Bos taurus*. Therefore, blood samples from Yak (*Bos grunniens*) and American Bison (*Bison bison*) were obtained during routine diagnostic blood sampling from a private farm in Bavaria and were made available by the veterinarian practitioners in charge: Dr. Christiane Grassl and Dr. Anton-Peter Grassl, Baierbach, administrative district of Landshut. Blood and milk samples from Water buffalo (*Bubalus bubalis*) were from the Farfengo Farm of the Labuaq buffalo milk company, Cremona, Italy and were made available through a DAAD-funded cooperation (Vigoni-Program) by Dr. Fabrizio Ceciliani, Dipartimento di Scienze Veterinarie e Sanità Pubblica Via Celoria 10, University of Milan, Milan, Italy.

These samples were also used in the semi-native Western blot procedure, as described in Chapter II (1.2.3 and 1.2.4). AdipoQ blood concentrations in Yak and Bison were estimated by ELISA; for their detection in Western blot, a concentration of 0.7 ng AdipoQ/lane (~ 1:250 dilution) was used. The serum and milk samples of Water buffalo were not detectable in ELISA because of missing parallelism and assay accuracy exclusively for Water buffalo samples; therefore, the Water buffalo serum was diluted 1:250 and the milk 1:10.

Semi-native Western blot analysis of blood and milk samples yielded signals in all samples. The MW patterns of AdipoQ in Yak, Bison, Water buffalo and buffalo milk were mostly the same (Fig. 17); a prominent MMW band (~ 130 kDa) and a HMW band (> 250 kDa) were detected in all samples. Generally, the pattern is comparable to that seen before in the serum and milk of *Bos taurus*.

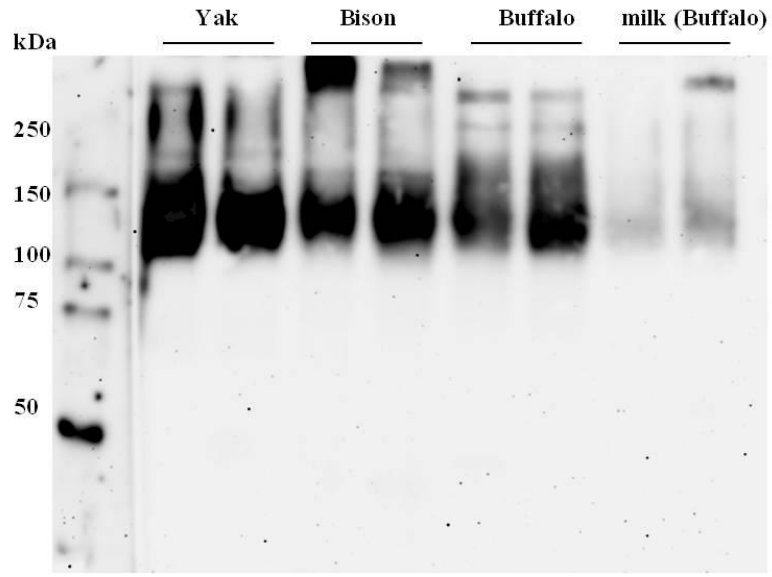

Fig. 17: Exemplary semi-native Western blot of serum and milk samples from different bovine species, applied in duplicate

The fact that Water buffalo could not be assayed for their serum and milk AdipoQ content by ELISA might be due to a matrix effect in the ELISA. The antibody detects AdipoQ in all three species of *bovidae* and the AdipoQ patterns in buffalo samples were not different to the others. Matrix effects are a common problem experienced by immunoassays, but few have been reported. Reasons for matrix effects are various; one possible factor is hindrance of an interaction between antigen and antibody (Lee et al., 2004). The estimated AdipoQ blood concentrations for Yak and Bison samples were well within the range of serum concentrations for Holstein dairy cows (Mielenz et al., 2013) i.e. 21.0 ± 0.4 µg/mL .

5. Adiponectin molecular weight patterns in allantoic fluid of dairy cows

The gene expression of AdipoQ and its receptors in human and rat placental tissue has been reported (Caminos et al., 2005). As there are significant differences between human and ruminant placenta types (haemochorialis vs. syndesmochorialis), the major source of circulating AdipoQ in neonates may differ in ruminants compared with humans. In addition, it was hypothesized that the estimated AdipoQ concentration in amniotic fluid is of fetal origin, because of the absent correlations with serum AdipoQ concentrations (Baviera et al., 2007). We aimed to characterize AdipoQ MW patterns and concentration in allantoic fluid (AF) and the corresponding serum of Holstein dairy cows. Samples of AF were collected during cesarean sections at the clinic for cattle in the University of Veterinary Medicine, Hanover, Germany from four Holstein dairy cows.

Directly after surgery, maternal blood samples were collected into EDTA tubes (Sarstedt AG & Co., Nümbrecht, Germany), centrifuged (1,000 x g, 15 min, 4°C) and then the plasma obtained was stored at -20°C until further analysis.

The mean plasma AdipoQ concentrations in cows subsequent to cesarean section were 19.8 ± 0.7 µg/mL, whereas the concentration in AF (2.6 ± 0.7 ng/mL) amounted to only 0.01% of the plasma values. There was no correlation between detectable AF concentrations and maternal plasma samples. The MW pattern of AdipoQ in AF was different from that in plasma (Fig. 18): the HMW form was the dominant form, whereas only faint bands were detectable in the MMW size range. In contrast, in the corresponding maternal plasma samples, both HMW and MMW were present.

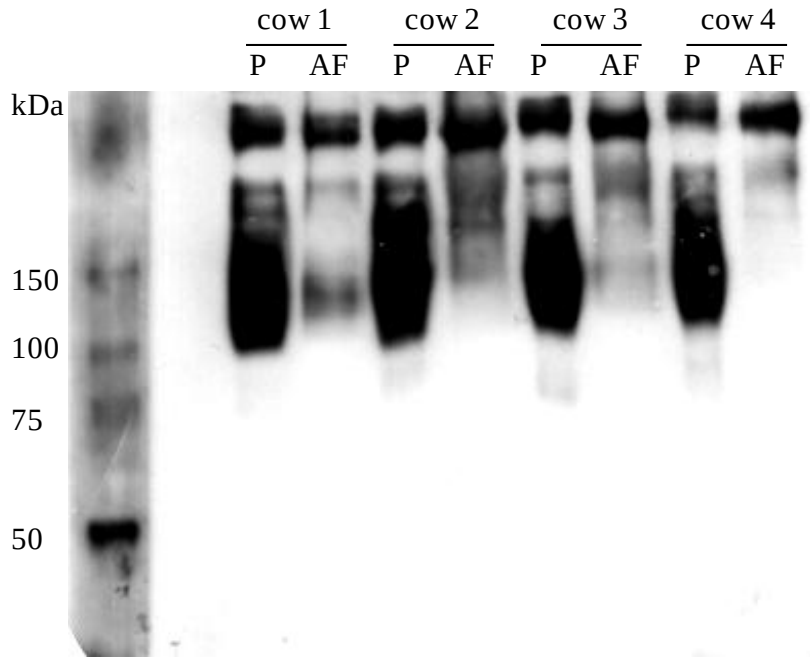

Fig. 18: Exemplary semi-native Western blot of AdipoQ molecular weight forms in maternal plasma (P) and allantoic fluid (AF) of four Holstein cows

Besides, we observed differences in MW of AdipoQ between AF and plasma. The HMW form of AdipoQ was present at higher concentrations in AF than in plasma; furthermore, HMW AdipoQ was the main band detectable in AF. Possible explanations might be that the protein is secreted by allantoic epithelial cells or could be of fetal origin. Supporting our explanation, incubated tissue explants of human placenta and amnion showed a basal release of AdipoQ (Lappas et al., 2005). In addition, PCR analyses of different fetal tissues in humans showed positive results for AdipoQ mRNA in amniotic membrane samples (Corbetta et al., 2005). Moreover, Corbetta et al. (2005) could not find a correlation between fetal and maternal serum AdipoQ concentrations.

With their findings, they hypothesized that the placenta does not contribute to fetal AdipoQ blood concentrations, which is in line with our findings. Possible explanations might include the fact that the protein is secreted by fetal tissue (Sivan et al., 2003).

Further applications of semi-native Western blot are given in Chapter IV.

CHAPTER IV: Manuscript (accepted by Theriogenology)

Characterization of adiponectin concentrations and molecular weight forms in serum, seminal plasma and ovarian follicular fluid from cattle

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Keywords:
adiponectin, seminal plasma, follicular fluid

ABSTRACT

Adiponectin (AdipoQ), an adipocyte-derived hormone, is one of the most abundant adipokines in the blood circulation. Adiponectin has various metabolic functions, such as improving insulin sensitivity in humans and in rodents. The role of AdipoQ in reproduction is not yet fully understood, but the expression of AdipoQ in reproductive tissues has been observed in various animals and humans, including chicken testis, bovine ovary and human placenta. The objective of this study was to characterize AdipoQ in the bovine body fluids related to reproduction. Therefore, we evaluated the seminal plasma (SP) from breeding bulls (n= 29) and follicular fluid (FF) from heifers (n= 14), and we also collected blood samples from these animals. In addition, blood samples from other bulls (n = 30) and heifers (n = 14) were assayed for AdipoQ. The concentrations were assessed using a bovine-specific ELISA, and the molecular weight (MW) pattern of the AdipoQ protein was estimated by Western blot (WB) analysis. The SP AdipoQ concentrations were approximately 180-fold lower compared to the serum concentrations. Furthermore, the AdipoQ concentrations in the serum and SP were positively correlated. The molecular weight patterns of AdipoQ in the serum and SP were different such that the high MW form of AdipoQ was more abundant in the SP than serum. The AdipoQ concentrations in the serum and SP also increased with age: Old bulls (> 6 years) had higher AdipoQ concentrations in the serum and SP than bulls ≤ 24 months of age ($P < 0.05$). In the FF, the AdipoQ concentrations were 1.6-fold lower than in the corresponding serum samples, and the concentrations in the serum and FF were not correlated ($P > 0.1$). In the FF, only the middle MW forms of AdipoQ were detectable by Western blotting. The MW pattern in the serum did not differ between the sexes. Our data provide both the AdipoQ concentration and MW patterns for bovine body fluids related to reproduction.

1. Introduction

Adiponectin (AdipoQ) is one of the most abundant adipokines that is present in the µg/mL range in the blood circulation. Adiponectin is predominantly secreted by adipocytes [1] and has potential benefits on insulin sensitivity [2] and also has anti-inflammatory activity [3]. Adiponectin exerts its effects by activating a range of different signaling molecules via binding to two transmembrane AdipoQ receptors, AdipoR1 and AdipoR2. AdipoR1 is expressed primarily in the skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver [4]. The major signaling molecules activated by AdipoQ are adenosine monophosphate-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38-MAPK) and the transcription factors peroxisome proliferator-activated receptor (PPAR) -α and nuclear factor kappa-light-chain-enhancer (NF-κB) [4]. AdipoR1 acts mainly via the APMK pathway, thus inhibiting fatty acid oxidation, whereas AdipoR2 acts through PPARα signaling, which stimulates fatty acid oxidation [5]. Adiponectin is synthesized as a single 28 kDa monomer but is not secreted as such; instead it undergoes multimerization to form different molecular weight (MW) multimers prior to secretion [6]. Adiponectin is found in the circulation in three different MW forms as follows: a trimer with low MW (LMW, 67 kDa), a hexamer with middle MW (136 kDa) and a multimeric form of 12 – 18 monomers with high MW (HMW, > 300 kDa). The AdipoQ monomer is only detectable after reducing and denaturation [6]. The HMW AdipoQ is considered the most biologically active form [7]. The potential effects of AdipoQ on reproduction have been addressed in various studies. Adiponectin may affect the female and the male reproductive system at different levels, through both central and local regulatory mechanisms. AdipoQ expression was observed in several species in different types of ovarian cells. Adiponectin mRNA was detected in chicken theca cells and, to a lesser extent, in the granulosa cells of follicles during different stages of maturation [8]. In cows, AdipoQ mRNA was detected in the theca, granulosa and cumulus cells as well as the oocyte [9]. In rat ovaries, the AdipoQ protein was immunohistochemically localized in theca-interstitial cells, corpus luteum and oocytes and lesser amounts were detected in granulosa cells [10]. By contrast, AdipoQ mRNA was reported to be absent/low in a human granulosa cell line as well as mouse and human granulosa and cumulus cells [10, 11]. Moreover, in situ hybridization localized the AdipoQ mRNA in adipose tissue adherent to the ovary but not in any ovarian cells in mice [11]. However, the expression of the AdipoQ receptors AdipoR1 and AdipoR2 was consistently confirmed in ovarian tissues from chicken, mouse, human and cattle [8, 10, 11].

AdipoQ may function in the ovary during steroidogenesis, although basal or FSH-stimulated steroidogenesis in primary rat granulosa cells was not affected by treatment with recombinant AdipoQ, and co-stimulation with insulin or insulin-like growth factor 1 (IGF-1) increased both progesterone and estradiol production [10]. By contrast, AdipoQ decreased insulin or IGF-1 induced progesterone production in bovine granulosa or theca cells [12, 13], and these inhibitory effects were primarily located in theca cells [12]. AdipoQ may also function in female reproduction during maturation of the oocyte; however, there are conflicting results. In cattle, adiponectin did not modify oocyte maturation in vitro, whereas the meiotic maturation of pig oocytes derived from follicles (3-6 mm diameter) was supported by recombinant porcine AdipoQ [14]. These results indicate that species differences may exist with regard to the specific ovarian response to AdipoQ.

For male reproduction, the AdipoQ protein was localized in the peritubular cells and Leydig cells of the chicken testis [15]. The Leydig cells were also positive for AdipoQ in the rat testis [16]. AdipoQ expression is less responsive to gonadotropins than metabolic signals, suggesting a function of AdipoQ as endocrine integrator linking metabolism and gonadal function [16]. AdipoR1 and AdipoR2 mRNA were observed in several testicular cells types and were higher in adult versus prepubertal chickens [15]. Androgens, particularly testosterone, are inversely related to the serum AdipoQ concentrations in humans [17] and mice [18], and treatment with recombinant AdipoQ inhibits testosterone secretion *ex vivo* [16]. Human seminal plasma (SP) is the male body fluid related to reproduction and reportedly contains AdipoQ at concentrations approximately 66-fold lower than serum. In addition, a positive correlation between the AdipoQ concentrations in both matrices was observed [19]. In human follicular fluid (FF), the concentrations of AdipoQ reach approximately 20 to 25% of the corresponding serum samples and are positively correlated with the serum values [20, 21]. In addition to the differences in AdipoQ concentrations, the isoforms of AdipoQ differ between the serum and FF. In the FF, the LMW form of AdipoQ is the most abundant fraction, whereas in the serum, the HMW fraction is predominant [22].

Studies of AdipoQ in cattle have been impeded by the lack of validated, species specific assays. There is one report of the serum AdipoQ concentration in bulls that were determined by an ELISA using an anti-human goat antibody [23]. However, the concentrations reported were approximately 100-fold lower than that expected based on the data published for humans and rodents [1].

The present study investigates the AdipoQ concentrations and MW patterns in bovine serum, FF and SP. Our objectives were as follows: (1) determine the AdipoQ concentrations and MW patterns in the serum and SP of breeding bulls and (2) characterize the AdipoQ in the serum and FF longitudinally during the estrous cycle in heifers.

2. Materials and methods

2.1. Bulls

2.1.1. Seminal plasma and blood samples

Blood and semen samples were collected at the same day from bulls owned by the bull stud center of the cattle breeding association "Rinder Union West e.G." in Borken, Germany. Blood samples were collected by jugular venipuncture from 59 bulls (Holstein: n = 56, Limousin: n = 1, Pinzgauer: n = 2; average age 37 ± 31 months (mean ± SD), median = 24 months, range = 11 months to 12 years). The samples were allowed to clot for 2 h before centrifugation (1,000 × *g*, 15 min, 4 °C), and the sera obtained were stored at -20 °C until further analysis.

The semen samples were obtained during the semen collection routine from 29 of the 56 Holstein bulls using an artificial vagina. One mL of the ejaculate collected from each bull was centrifuged (10,000 × *g*, 10 min, 4 °C), and the supernatant (SP) was stored at -20 °C.

2.1.2. Estimation of sperm characteristics

The volume of each ejaculate was recorded by weighing, and the density was estimated by photometry. The motility of the spermatozoa was estimated using Computer Assisted Sperm Analysis (CASA) with SpermVision™ (Minitüb GmbH, Tiefenbach, Germany). The variables characterizing the motility were total motility (tMOT), progressive motility (pMOT) and local motility (lMOT). In addition, the percentage of dead cells (not moving) was recorded. For 2 of the 29 ejaculates obtained, the motility data were not available. The pMOT was > 75% for all bulls (85.1 % ± 19.0%, means ± SD, range 77.0 – 96.1%), except for 3 bulls that had *azoospermia.*

2.2. Female animals and sample collection

2.2.1. Follicular fluid

Simmental heifers (n = 14; 15-20 months old) from the Training and Research Center Frankenforst, of the University of Bonn were synchronized by intramuscular (i.m.) administration of 500 µg cloprostenol ($PGF2_{\alpha}$, Estrumate®; Essex Tierarznei, Munich, Germany) twice within 11 d. Two days after each $PGF2_{\alpha}$ treatment, the animals received 10 µg GnRH (i.m.) (Receptal®; Intervet, Boxmeer, The Netherlands). The third day after the last $PGF2_{\alpha}$ injection was day 0 of the estrous cycle.

The blood samples and ovaries were collected from animals sacrificed at d 3 (n = 6), 7 (n = 3) or 19 (n = 5) after the onset of estrus as confirmed by rectal palpation by an experienced veterinarian. Only heifers with a contracted uterus at day 0 were classified as in estrus. The follicular fluid was collected from the dominant follicle (8-12 mm diameter) from each animal. The blood samples were centrifuged at 1,500 × *g*, 4 °C for 20 min after clotting for 2 h. The serum and FF were stored at -20 °C until further use.

To determine the serum concentrations during the estrous cycle in more detail, serum samples were obtained on days 0, 3, 6, 10, 12, 14, 17 and 19 of the estrous cycle from 14 additional Simmental heifers (24 - 28 months old) synchronized as described above.

2.3. Assessment of the concentrations and molecular weight forms of adiponectin

2.3.1. Quantitative assessment of the adiponectin concentrations by ELISA

The serum, SP and FF were analyzed in duplicate for AdipoQ using a competitive ELISA developed in-house as described previously [24]. Assay accuracy was confirmed by establishing the linearity of serially diluted samples. The range of the assay was 0.07-1.0 ng/mL, and the limit of detection was 0.03 ng/mL. The intra- and interassay coefficients of variation (CVs) were 7 and 9%, respectively.

2.3.2. Western Blot

Western blot analysis was performed to determine the different molecular weight forms of AdipoQ in the different body fluids. The samples were used without reducing and heat-denaturing.

2.3.2.1. Sample preparation

The AdipoQ concentrations assessed via ELISA were used to standardize the amount of sample subjected to electrophoresis and Western blotting for the same AdipoQ concentrations. Prior to the comparative analyses, the amount of AdipoQ per lane allowing for optimal display of the different MW forms in each body fluid was tested and optimized. The final concentrations of the fluids were standardized as follows: serum 1 ng; SP 0.5 ng; and FF 1 ng. Approximately equal concentrations of AdipoQ per sample were loaded by diluting the samples with PBS

2.3.2.2. Western blot procedure

The diluted samples were mixed with sample buffer (final concentration: 0.064 M Tris HCl pH 6.8, 1% SDS, 0.01% bromophenol blue, and 10% glycerol). Before loading on 8% SDS gels, the samples were centrifuged for 5 min at 10,000 × *g*, 4 °C. The proteins separated by SDS-PAGE were transferred onto polyvinylidene diflouride membranes (PVDF) (GE Healthcare Europe, Freiburg, Germany) using tank blotting with the Criterion Blotter System (Bio-Rad Laboratories, Munich, Germany). After blotting, the membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST) and 10% Roti®-Block (Carl Roth, Karlsruhe, Germany) for 60 min at room temperature (RT). The membranes were exposed to primary antibody (0.25 µg/mL) (same as the ELISA) for 1 h at RT and washed 4 times with TBST containing 0.05% Tween 20. Similarly, the membranes were treated with a secondary goat-anti-rabbit antibody conjugated with horseradish peroxidase (1:100,000; SouthernBiotech, Birmingham, AL, USA). After washing, the immune complex was detected with an enhanced chemiluminescence detection system (GE Healthcare) using CL-XPosure film (Thermo Scientific, Munich, Germany). The MW of the bands detected was assessed by comparison with a MW marker (Precision Plus Protein™ WesternC™ Standards, BioRad). Where appropriate, the intensity of the bands was quantified densitometrically using the VersaDoc MP4000 imaging system with Image Lab software (Bio-Rad, Munich, Germany), and the ratio between the intensities of the bands corresponding to the MMW and HMW was calculated.

2.4. Statistical analyses

All statistical analyses were performed using SPSS (IBM SPSS Statistics 21, Ehningen, Germany). The data are presented as the means ± SEM, and significance was P-values < 0.05, whereas P < 0.1 was considered a trend. The normal distribution of all data was tested using the Kolmogorov-Smirnov test.

The AdipoQ concentrations in the serum and SP were compared between each of the 3 age and 3 motility groups (described below) by non-parametric Mann-Whitney-U test. Testing for correlations between different variables was performed with either Pearson correlation for normally distributed data or Spearman Rho correlation when the data were not normally distributed.

To test for potential temporal differences throughout the estrous cycle, a general linear model with repeated measurement was used. The model included pair-wise comparison with Bonferroni correction to evaluate differences between the individual days of the estrous cycle.

To test for the potential effects of age, the samples obtained from the bulls were classified as follows: bulls ≤ 24 months of age (n = 30; 16 ± 1 months of age, average body weight 446 ± 20.7 kg), bulls > 2 and ≤ 6 years (n = 22; 44 ± 4 months, 891 ± 45.4 kg) and bulls > 6 years (n = 8; 102 ± 22.4 months, 1050 ± 40.5 kg).

To assess potential differences in the MW pattern from the samples obtained from bulls with different sperm quality, pools of both serum and SP were generated from animals based on their different progressive spermatozoa motility (pMOT). Groups were generated as follows: bulls with ≥ 90% pMOT (n = 14; 93.4 ± 0.4%), bulls ≥ 80% and < 90% pMOT (n = 5; 86.8 ± 0.8%), bulls < 80% pMOT (n = 5; 77.1 ± 2.86%) and azoospermic bulls (n = 3).

3. Results

3.1. Adiponectin concentrations and MW patterns in bull sera and seminal plasma

The AdipoQ concentrations in the serum (31.1 ± 1.42 µg/mL) were higher (P < 0.001) than the SP (0.18 ± 0.01 µg/mL). The serum and SP AdipoQ concentrations were correlated as shown in Fig. 1. The mean ratio between the serum and SP AdipoQ was 180 ± 41. The serum AdipoQ concentrations from the 3 non-Holstein bulls were well within the range described here.

Comparison of the AdipoQ concentrations in the serum and SP between the different age groups showed that the oldest bulls (>73 months) had higher concentrations than the young (≤ 24 months) and middle aged (25-72 months) animals (Fig. 2).

For all age groups, the serum and SP concentrations were correlated with age ($r = 0.458$, $P < 0.05$ and $r = 0.380$, $P < 0.05$, respectively; Spearman-Rho correlation). However, when the correlations were calculated within the age groups, the correlations between the serum and SP AdipoQ were limited to the young (n = 13) and middle aged (n = 10) animals ($r = 0.752$ and $r = 0.807$, $P < 0.01$, respectively), whereas for the old bulls (n = 6), no such relationship ($r = 0.551$, $P = 0.257$) was observed. The ratio between the serum and SP AdipoQ concentrations was not different between the age groups. Based on the various semen characteristics recorded, no relationship was observed for the AdipoQ concentrations in the serum or SP. The only exception was the group of old bulls in which progressive and total sperm motility were inversely correlated with the serum AdipoQ concentrations ($r = -0.886$, $P < 0.05$). In general, sperm motility increased with age.

The seminal plasma differed from the serum for the MW patterns of AdipoQ (Fig. 3). The intensity of the HMW band in SP was approximately 5-fold higher than in serum. The ratio between the MMW and LMW AdipoQ bands was lower in the SP than in serum.

3.2. Adiponectin concentrations and MW patterns in heifer sera and in follicular fluid

The adipoQ concentrations in the serum and FF obtained from the dominant follicle remained fairly constant during the estrous cycle (Fig. 4A). On average, the AdipoQ concentrations in the FF (19.4 ± 1.4 µg/mL) were approximately 60% of serum (31.8 ± 1.5 µg/mL). The serum and FF AdipoQ concentrations during the estrous cycle were not correlated ($r = -0.434$, $P > 0.1$, n=14, Pearson correlation). When blood samples were repeatedly collected at higher frequency from additional animals across the estrous cycle, a variation ($P < 0.01$) over time was observed (Fig. 4B). On day 10 of the estrous cycle, the lowest concentrations of AdipoQ were observed, which were approximately 1.2-fold lower than on day 3 and day 0 ($P < 0.046$ and $P < 0.009$, respectively).

The MW patterns of AdipoQ in the FF differed from the serum as shown in Figure 5. The HMW band was virtually absent in the FF independent of the stage of the estrous cycle.

3.4. Adiponectin serum concentrations in male versus female cattle

The mean serum concentrations of AdipoQ differed between the heifers and bulls of approximately the same age, at 15 to 28 months. The bulls had lower ($P = 0.002$) serum AdipoQ concentrations than the heifers (25.1 ± 0.9 vs. 31.0 ± 1.5 µg/mL).

4. Discussion

The presence of AdipoQ in bovine SP and FF was detected. Using a bovine specific ELISA developed in-house [24], the AdipoQ concentrations in the SP and FF were quantified and compared with the serum values. The applicability and validity of this ELISA for SP and FF was confirmed by parallelism of serial dilutions with the standard curve. In both the SP and FF, the AdipoQ concentrations were lower than in the corresponding serum samples. Western blot analysis was used to characterize the MW forms of the AdipoQ protein in SP and FF and yielded divergent MW patterns in the different body fluids.

Adiponectin in seminal plasma and serum of bulls

The AdipoQ concentrations in the SP were approximately 180-fold lower than the corresponding serum concentrations. Thomas et al. [19] reported that the AdipoQ concentrations in the SP of men were approximately 66-fold lower than the concentrations in serum and were correlated. The authors suggested that AdipoQ is transferred from the blood to testis tissue, particularly via gaps in the blood testis-barrier. The correlation (Spearman-Rho) observed in the current study between the serum and SP AdipoQ concentrations in bulls supports the hypothesis that SP AdipoQ originates from the blood.

The lack of correlation between the serum and SP concentrations in the old bulls may be explained by the small number of animals in this group. However, the MW patterns of AdipoQ in bull sera and SP were different. In the serum, the MMW was the primary band, whereas only a faint band for the HMW was observed. Similar MW patterns of the AdipoQ protein in serum were shown for dairy cows [24, 25, 26]. In contrast to the serum, the SP exhibited a prominent HMW band in addition to the MMW band, which was also present. The composition of SP is affected by its production sites, including the rete testis, epididymis, and accessory sex glands [27], which may differ in their blood-tissue barrier functions. In addition, the proximity to adipose tissue, particularly visceral adipose tissue, may also contribute to the relatively higher portion of HMW AdipoQ in SP. The ratio of HMW/total AdipoQ was higher in visceral adipose tissue, which also comprises gonadal fat, than in subcutaneous fat [28]. Moreover, SP in contrast to FF, is not a transudate from the blood but results from secretion processes at its various sites of production. The composition of SP deviates from blood serum in many respects, and the concentration of anorganic and organic constituents may affect the formation of AdipoQ multimers. For example, the concentration of calcium in bovine SP is approximately 3 times higher than in the blood serum [29], and calcium promotes the formation of HMW AdipoQ [30].

SP AdipoQ may function on spermatozoa because AdipoR1 and R2 mRNA is expressed in bull spermatozoa [23]; however, the relationships between the SP AdipoQ concentrations and functional sperm characteristics were not established in this study for either bulls or humans [19].

Age dependent differences in adiponectin concentrations

The increase of AdipoQ concentrations in the serum with age for bulls is consistent with observations in aging humans [31, 32]. In men, this age-related increase of serum AdipoQ concentration is more pronounced than in women. Our results are limited to bulls because the age range of the heifers here was 15 to 28 months. However, when comparing cows up to 4 lactations from previous studies, the AdipoQ serum concentrations were greater in multiparous than primiparous lactating cows from day -21 pre partum until day 252 during lactation [33]. One possible explanation for the increase of AdipoQ serum concentrations with age in bulls may be changing sex hormone concentrations over the lifespan. Testosterone concentrations decrease with increasing age in men [34], and testosterone and 5α-dihydrotestosterone suppress AdipoQ secretion from cultured adipocytes [18]. However, inconsistent results regarding the influence of AdipoQ on testosterone and vice versa are published. For example, testosterone and AdipoQ are positively correlated in men [35], whereas in rats, a negative correlation was found [36].

In bulls, no increase in testosterone with age was detected [37] in animals 8.5 to 18 months of age, which is equivalent to the age of the young bulls in the present study. By contrast, a recently published study showed a highly positive correlation between the serum AdipoQ and testosterone concentrations in bulls. The increase of serum AdipoQ concentrations with increasing fertility was similar to the testosterone concentrations [23]. However, the absolute serum AdipoQ concentrations in the aforementioned study were approximately 100-fold lower than for monogastric species and cows [1, 24]; therefore, the values cannot be directly compared.

In addition, we observed lower serum AdipoQ concentrations in young bulls (< 24 months of age) than young cows of about the same age. However, we cannot rule out that this difference may be attributable to different environmental conditions and different breeds because the bulls were primarily Holstein Frisians, whereas the heifers were all Simmental). The body fat content, which depends on both the breed and sex, may also contribute to this observation. Interestingly, for the bulls, the correlations between the serum and SP AdipoQ differed between the age groups.

The oldest bulls showed no correlation between the serum and SP, whereas in the young and middle aged bulls, high correlations ($r = 0.752$, $r = 0.807$, respectively, $P < 0.05$, Spearman-Rho correlation) were observed. However, the missing correlation in the old bulls may be because of the few ($n = 6$) animals in this group. The correlations within the 2 other age groups and across all bulls further supports that AdipoQ from the blood may be the main source of AdipoQ in the SP.

Adiponectin in the serum and FF during the estrous cycle

The serum AdipoQ concentrations varied during the estrous cycle and were lowest at day 10 in the animal group that was repeatedly sampled. In cattle, the serum concentrations of progesterone increase from estrus to day 10 and then plateau until luteolysis, whereas appreciable increases of estradiol-17ß are limited to the time of estrus [38]. Increasing progesterone concentrations, although not assessed here, may be related to the decrease in serum AdipoQ at day 10 of the estrous cycle because progesterone treatment decreases AdipoQ mRNA expression in the inguinal fat of female rats [39].

The concentrations of total AdipoQ in the FF from heifers were on average 62% of the corresponding serum samples. In swine (prepubertal gilts), the AdipoQ concentrations in the FF were 80 - 90% of the serum [40]. It should be noted that all data for the AdipoQ concentrations in human FF are from patients undergoing ovarian stimulation, such that the samples were obtained after gonadotropin treatment.

The concentrations of total AdipoQ in these FF samples as 22-29% of the serum values and were strongly correlated to each other [20, 22]. For the heifers in our present study, the AdipoQ concentrations in the blood serum and FF were not correlated, but this may be because of the relatively few animals from which both FF and blood were obtained in parallel. In the bovine ovary, AdipoQ and its receptors are expressed at both the mRNA [9] and protein levels [13]. The presence of AdipoQ mRNA in bovine oocytes, theca, cumulus and granulosa cells supports the hypothesis of local production sites for FF AdipoQ. AdipoQ mRNA was expressed in the adipose tissue adherent to the mouse ovary [11], this adipose tissue is a likely source of AdipoQ in the FF. Adiponectin mRNA expression varied throughout follicular development, and the granulosa and cumulus cells of large follicles had 2-fold higher expression than small follicles [9]. However, we did not observe any differences in the FF AdipoQ concentrations between small and large follicles in ovaries collected from two cows at a local abattoir (data not shown). The divergent MW pattern we observed in the FF versus serum indicating that there was relatively less HMW AdipoQ in the FF is consistent with reports in humans.

The percentage of HMW adiponectin in human FF was 11% of that in serum versus 21% and 45% of the MMW and LMW, respectively [22]. The follicular fluid is a product of both the transfer of blood plasma constituents across the blood follicular barrier and the secretory activity of granulosa and theca cells [41]. The local secretion of AdipoQ from ovarian cells into the FF may occur [13], and transudation of AdipoQ from the blood to FF has been previously suggested [21]. For the latter, the relative concentrations of the different MW forms in the FF versus serum may be altered because the amount of proteins in the FF depends on the MW of the protein, and the amount decreases as the MW increases [42].

4.1. Summary and conclusions

We have demonstrated that the AdipoQ concentrations in the SP and FF are lower than the corresponding blood values. Furthermore, the MW patterns of the AdipoQ varied between the SP and FF but not between serum samples. In addition, no sex-related differences in the MW patterns were observed. Correlations between the reproductive body fluids and the corresponding blood values were established for the SP only, but due to the limitations in the number of females studied, correlations may also exist for the FF. Therefore, we conclude that the AdipoQ concentrations in the SP are likely blood borne and originate from the adipose tissue, and the potential contribution of local secretion from the testes, if any, is only marginal. For the AdipoQ present in the bovine FF, transudation from the blood but also local secretion is feasible.

In the present study, increasing age was associated with increasing serum and SP AdipoQ concentrations in bulls. The functional importance of AdipoQ in the reproductive fluids remains to be elucidated.

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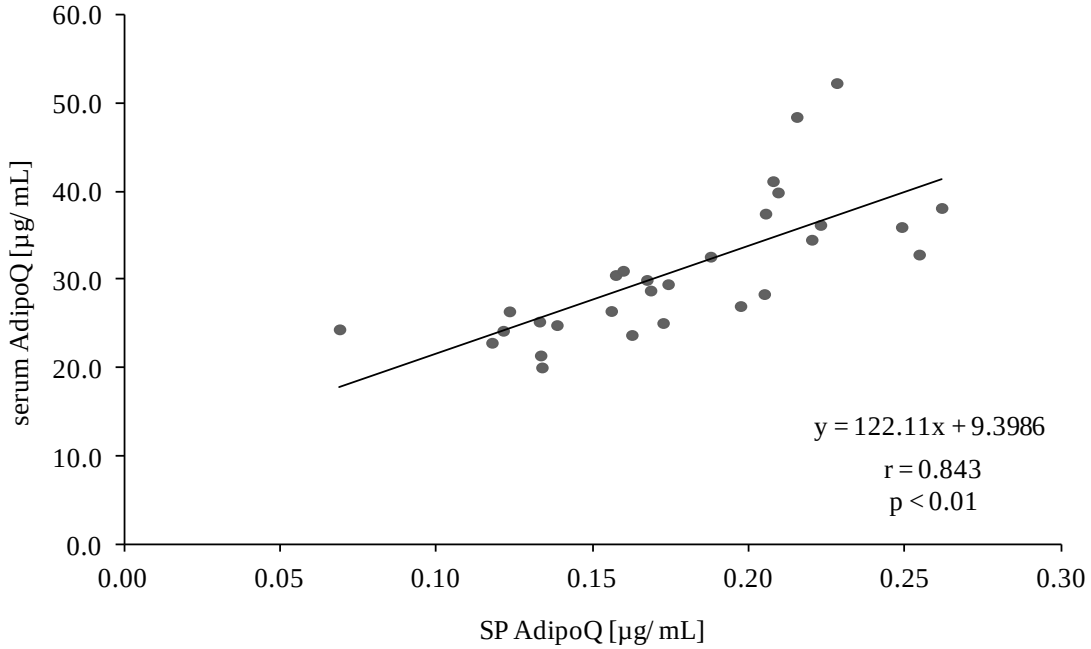


Figure 1: Correlation of serum and seminal plasma (SP) adiponectin (AdipoQ) [µg/mL] concentrations in Holstein breeding bulls (n = 29)

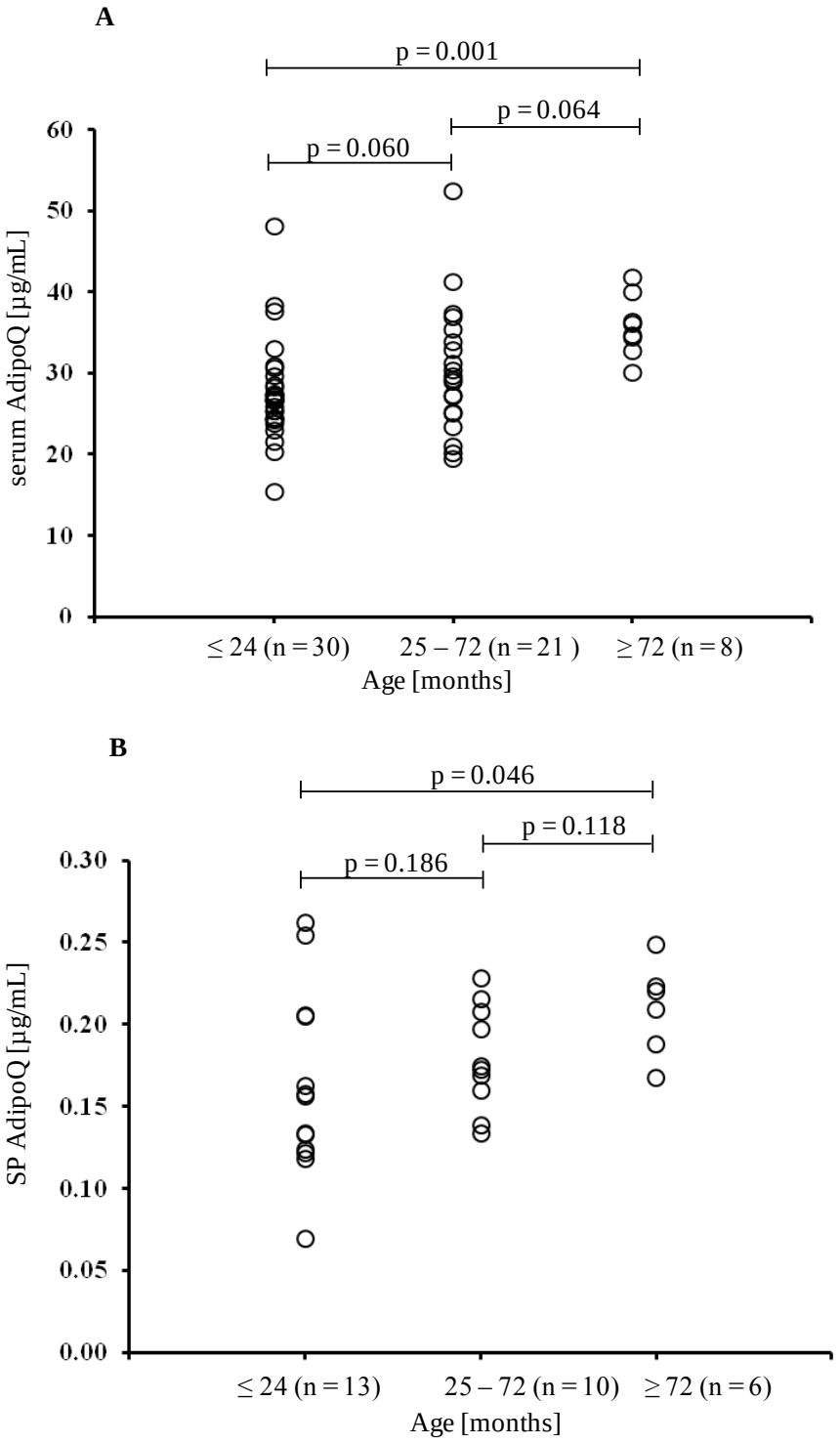


Figure 2: Scatter plots of the adiponectin (AdipoQ) concentrations in serum (A, n = 59) and in seminal plasma (SP, B, n = 29) of breeding bulls classified according to their age in 3 different groups

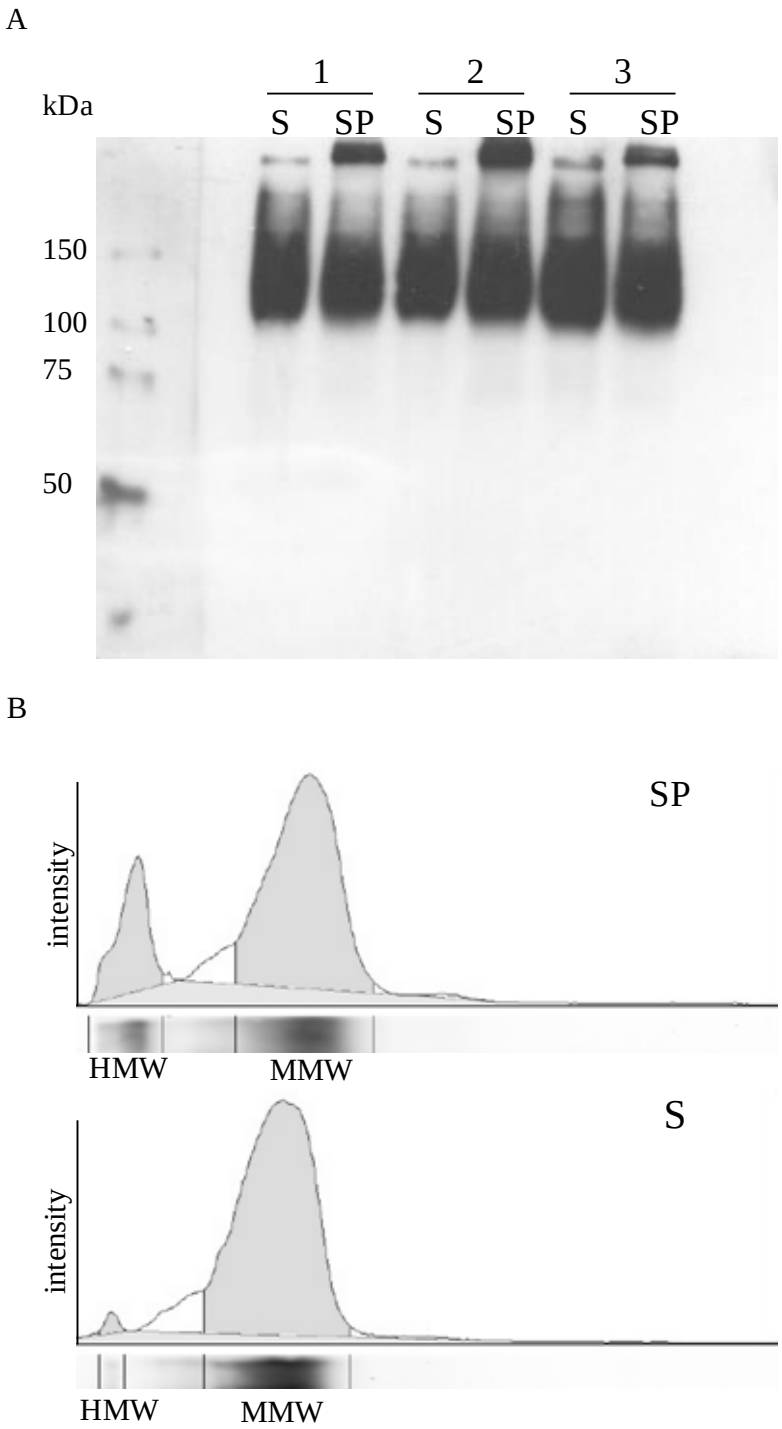


Figure 3: Molecular weight patterns of adiponectin (AdipoQ) in bulls` serum (S) and seminal plasma (SP). (A) Exemplary Western blot of AdipoQ multimeric isoforms under non-reducing and non heat-denaturing conditions. Samples were proportionately pooled from groups classified according to the progressive motility (pMOT) of spermatozoa. **1**: bulls with ≥ 90% pMOT (n = 14: 93.4 ± 0.4%), **2**: bulls with ≥ 80% and < 90% pMOT (n = 5: 86.8 ± 0.8%) and **3**: bulls with < 80% pMOT (n = 8: 48.2 ± 13.2%). (B) Exemplary lane profile of serum and SP samples showing different intensities in high molecular weight (HMW) and middle molecular weight (MMW) bands

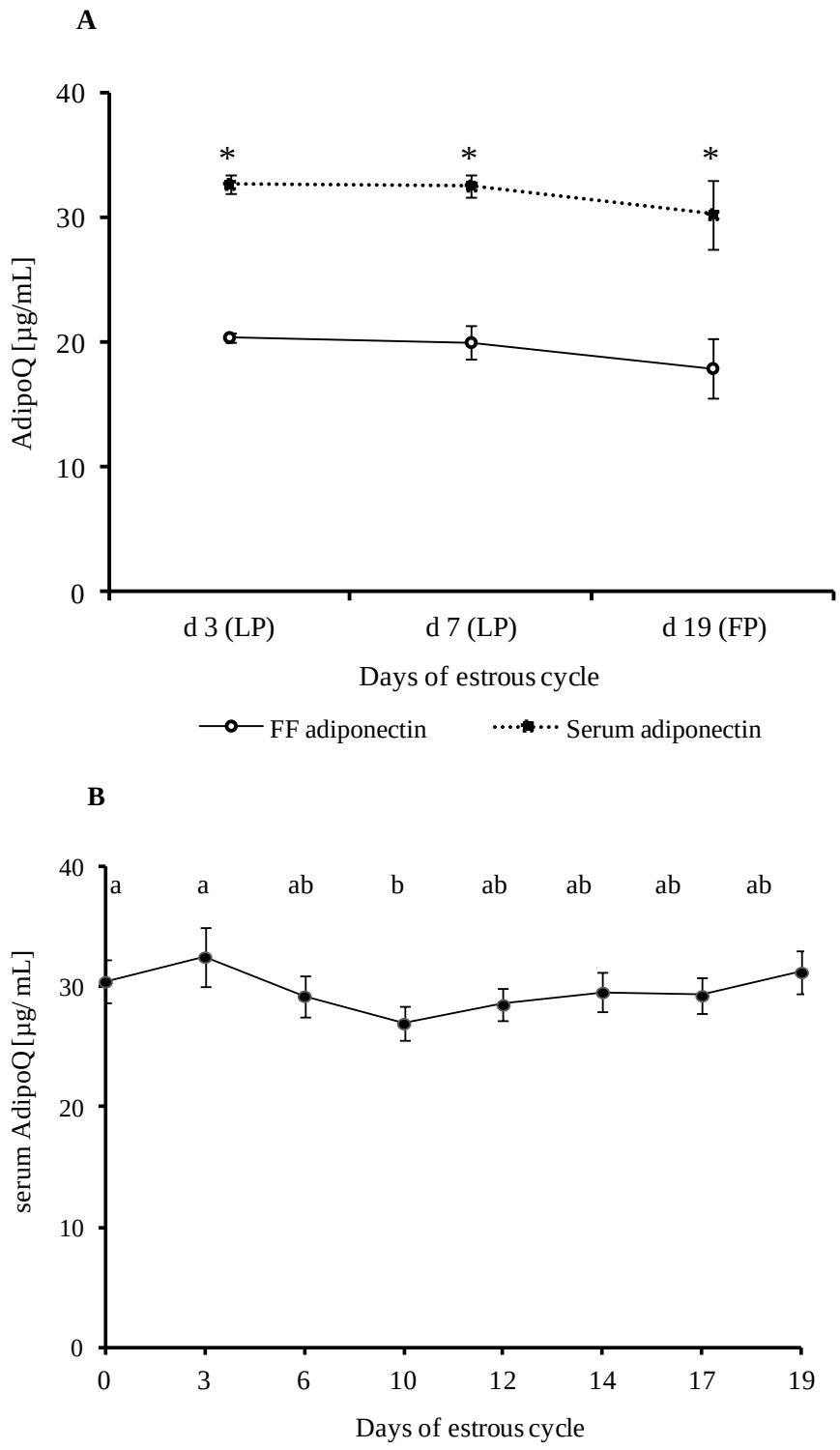


Figure 4: Changes in adiponectin (AdipoQ) concentrations (means ± SEM) during estrous cycle in follicular fluid (FF) and in serum.
(A) Adiponectin concentrations in follicular fluid (FF) and in serum from Simmental heifers (n = 14) sampled at targeted slaughter at 3 different days of the estrous cycle. LP = luteal phase; FP = Follicular phase. *: $P < 0.05$ for differences between serum and FF AdipoQ concentrations.
(B) Changes in serum AdipoQ concentrations of Simmental heifers (n = 14) sampled repeatedly during the estrous cycle; different letters indicate differences ($P < 0.05$) between time points

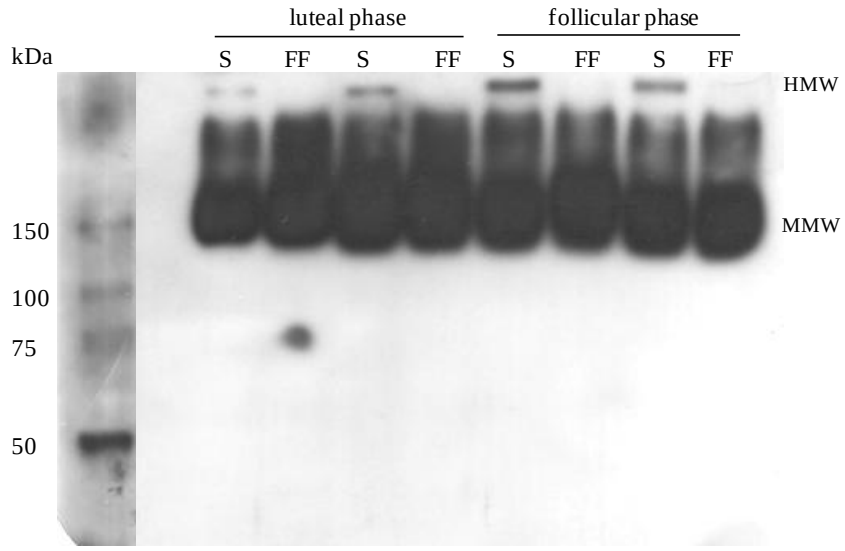


Figure 5: Exemplary Western blot of adiponectin (AdipoQ) multimeric isoforms under non-reducing and non heat-denaturing conditions in serum (S) and in follicular fluid (FF) of 4 heifers, 2 in luteal phase and 2 in follicular phase (day 3 and day 19 of the estrous cycle, respectively). High molecular weight (HMW) and middle molecular weight (MMW) bands

CHAPTER V: Recombinant production of adiponectin and functional studies

Recombinant proteins are biotechnologically produced proteins which were expressed by genetically modified bacteria, yeast, insect cells or mammalian cells. With the use of recombinant protein production in *E. coli,* a fast and high yield method for protein expression was developed. To purify proteins from crude extracts, affinity tags could be used as highly efficient tools. Affinity tags are peptide sequences which are genetically grafted onto a recombinant protein. The advantage of using fusion proteins to facilitate the purification and detection is well-recognized and many different purification protocols are available (Terpe, 2003). Recombinant proteins are often used for antibody generation, as standards in quantitative detection systems or for the investigation of functional effects.
In the following chapter, the expression of recombinant AdipoQ linked with an affinity tag, its purification and testing of its effects on lymphocyte proliferation are described.

1. Material and methods

1.1. Vector generation with IBA Star Gate Cloning

Adiponectin was produced by a combinatorial cloning procedure from IBA bioTAGnology GmbH, Göttingen, Germany. The cloning procedure was carried out according to the manufacturer's instructions.

1.1.1. Donor vector generation

Beginning with the amplification of the AdipoQ gene, cDNA from bovine sc. AT was used to amplify the AdipoQ gene by PCR. Additionally, the AdipoQ gene was equipped at both termini with combinatorial sites, the StarCombinase recognition area and a cleavage site for factor Xa. The StarCombinase recognition is important for the oriented insertion of AdipoQ into the entry vector. The Primers (Tab. 2) were designed using the StarPrimer D'Signer software, as recommended in the manual.

Table 2: Characteristics of the primers

Name	accession number	Sequence	Length of PCR product (bp)
Bos taurus AdipoQ	NM_174742.2	forward: 3'AGCGGCTCTTCAATGATTGAGGGTCGCGAGGACAACATGGAAGATCC'5 reverse: 5'AGCGGCTCTTCTCCCTTCAACAATGTTATGGTAGAG'3	711

After the amplification of the AdipoQ gene by PCR, the PCR product was checked for size and quality in an agarose gel (1.0%). The size of the PCR product was estimated by comparing the band size with those of the marker ΦX174 DNA/BsuRI (HaeIII) Marker, 9 (Thermo Fisher Scientific, Waltham, MA, USA).

The second step in the cloning procedure was purification of the PCR product using a commercial kit (innuPREP DOUBLEpure Kit, Analytikjena, Jena, Germany). Briefly, two aliquots of the PCR product (100 µl in total) were mixed with 1000 µl binding buffer and mixed. The purification column was loaded with the mix of buffer and PCR product, centrifuged (10,000 x *g*, 2 min, RT) and then loaded again until the PCR product was used up and the binding of the PCR products had been completed. The elution of the PCR products was achieved as follows: 18 µl elution buffer were loaded onto the column and incubated for 1 h at RT. Finally, the PCR products were harvested by centrifugation (8,000 x *g*, 1 min, RT), and the flow-through was collected and used for further cloning procedures.

The third step of donor vector generation was insertion of the amplified AdipoQ DNA into the entry vector. The entry vector was provided in a reaction tube into which 2 nM of the AdipoQ DNA and the StarSolutions M1, M2 and M3 had to be added. After gentle mixing, the tube was incubated for 1 h at 30°C. Meanwhile, the competent *E. coli* Top 10 cells were thawed. After incubation, the transformation into *E. coli* Top 10 cells was achieved by adding 10 µl of the entry vector reaction mixture to the cells. Afterwards, the tube was incubated for 30 min on ice, then gently mixed, incubated for 5 min at 37°C, mixed again and incubated for a further 3 min on ice. After transformation of the entry vector into *E. coli* cells, 900 µl Luria Bertani (LB) (Carl Roth, Karlsruhe, Germany) medium were added and a further incubation of 45 min at 37°C followed. To culture the transformed *E. coli* cells, 100 µl of the cell suspension were plated in duplicate on an LB agar plate which contained 50 mg/L kanamycin and 50 mg/L X-gal (AppliChem, Darmstadt, Germany).

Finally, these plates were incubated at 37°C overnight. The next morning, a liquid culture and a master plate were made. Single colonies from the agar plate were picked and transferred either into a tube containing LB-medium or on an LB-agar (Carl Roth, Karlsruhe, Germany) plate.

1.1.2. Verification of the correct insertion of adiponectin by restriction analysis

The transformed bacteria, from the liquid night culture, were used for restriction analysis. First, the DNA was extracted by the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), according to the instructions provided in the manual. The procedure is based on the alkaline lysis of bacterial cells and is followed by adsorption of the DNA onto a silica membrane. Three basic steps were necessary, beginning with preparation and clearing of the bacterial lysate. Then, the DNA was absorbed onto the QIAprep membrane and the plasmid DNA was finally washed and eluted.

The extracted plasmid DNA concentration was estimated by NanoDrop™ (PEQLAB Biotechnologie GMBH, Erlangen) measurement.

The restriction analysis was carried out with 1 µg of extracted DNA and the restriction enzymes XbaI and HindIII (Thermo Fisher Scientific). The restriction was necessary to confirm the insertion of AdipoQ DNA into the entry vector. XbaI recognizes T^CTAGA sites and HindIII A^AGCTT sites in DNA at a temperature optimum of 37°C. The samples were prepared with Tango Buffer (Thermo Fisher Scientific), DNA and both restriction enzymes. The restriction program was run in a thermal cycler (Peltier Thermal Cycler, PTC 200, MJ Research, Watertown USA) for 4 h at 37°C, followed by 20 min at 80°C to stop the enzyme activity, and samples were held at 4°C until the samples were taken out of the cycler.

Verification of the restriction analysis was done by electrophoresis with a 1% agarose gel. Additionally, the sequence of the obtained PCR product had to be confirmed, since PCR may lead to mutations and improper product ends. Therefore, the restricted plasmid, with the appropriate primers provided in the cloning kit, was sent to a sequencing company (SeqLab, Sequence Laboratories GmbH, Göttingen, Germany).

1.1.3. Destination vector generation and transformation in *E. coli*

After sequence confirmation of the AdipoQ insert, the procedure was continued by cloning the donor vector plasmid into different acceptor vectors (see table 3) to generate the final destination vectors. The use of different tags provided the possibility to select the optimal combination of the expression patterns of the protein (soluble form or inclusion body) and to choose a specific protein purification system. The appropriate acceptor vector was mixed with the donor vector and with StarSolutions A1 to A3 followed by a short incubation (for 1 h at 30°C).

Table 3: Different affinity tags of the acceptor vectors

Acceptor vector	**IBA 4**	**IBA 35**	**IBA 25**	**IBA 105**
N-terminal Tag	Strep Tag + signal sequence	6 His Tag	GST Tag	One strep
Tag Size (bp)	106	32	653	104

Strep: synthetic streptavidin; 6 His: polyhistidine-tag; GST: Glutathione S-transferase; One strep: tandem Strep tag.

Afterwards, the destination vector was transformed into *E. coli* Top 10 cells, as described in 1.1.1. To verify the insertion of AdipoQ into the destination vector, the cells were plated on LB agar plates containing ampicillin and X-gal. Blue/white staining was used as a transformation indicator. The desired destination vector, including the AdipoQ gene, will generate white colonies, whilst non-desired acceptor vectors, i.e. those not bearing the target gene insert, will generate blue colonies. This is caused by the insertion of the LacZ gen in the acceptor vectors, which produces blue stained colonies in the presence of X-gal. If AdipoQ is inserted properly, the LacZ gene is replaced and the colonies stay white. Finally, white colonies were taken for overnight culture. Subsequently, the insertion of AdipoQ was verified by restriction analysis, as explained in 1.1.2.

1.2. Protein overexpression in *E. coli*

First, a pre-culture was prepared with 100 mL LB media containing 100 mg/mL Ampicillin (AppliChem). Transformed *E. coli* were taken either directly from a colony on an agar plate or from a previously prepared glycerin stock. A small amount of cells was sufficient for the

pre-culture. A pipette tip was dipped into the colony and put into the LB media flask. Growing of the cells was achieved overnight (16-18 h) under shaking (350 rpm) at 37°C.

The next morning, the main culture was prepared in four Erlenmeyer flasks with 200 mL LB media each, supplemented with 100 mg/mL Ampicillin. The main culture was inoculated by gradually pipetting the pre-culture into the freshly prepared LB media until an optical density (OD) of 0.1 was reached at 578 nm. The culture was incubated for at least 1h before the next measurement of growth of the culture. The aim was to reach an OD between 0.4 and 0.5. If 0.5 was reached, a zero sample was taken, centrifuged (5000 rpm, 5 min) and then the pellet was resuspended in 20 µl SDS-PAGE sample buffer (5X) and frozen (-21°C) until further use. Out of each fresh culture, a glycerin stock (300 µl glycerin + 500 mL culture) was generated and stored frozen at -21°C.

The overexpression of AdipoQ by *E. coli* Top 10 cells was induced by adding 200 µl Anhydrotetracycline (200 ng/mL) (AppliChem, Darmstadt, Germany) to the main culture. To check the expression level of AdipoQ, a sample of 1 mL was taken hourly over a duration of 4 h. These samples were centrifuged (5000 rpm, 5 min), the supernatant was drained off and the pellet was resuspended in 20 µl SDS-PAGE sample buffer (5X) and frozen (-21°C) until further use in SDS-PAGE. After 4 h, the overexpression was stopped by centrifugation (5000 rpm, 15 min, 4°C) of the whole culture medium. The supernatant was collected in a tube and frozen (-21°C) until further use in SDS-PAGE; the remaining pellet was treated likewise.

1.3. Protein purification

1.3.1. Sonication procedure

Sonication was used to identify whether the recombinant AdipoQ remained soluble in the cytoplasm or was accumulated in insoluble aggregates.

The residual cell pellets from the expression were thawed on ice, weighed and resuspended in Lysis- Equilibration-Washing (LEW) Buffer. The pellets were dissolved in 20 vol. of LEW Buffer. Afterwards, the cell solution was sonicated (Sonoplus SH 70G, Bandelin electronics, Berlin, Germany) three times for 1 min on ice followed by high speed centrifugation (18,000 x *g*) for 30 min at 4°C. The resulting supernatant was drained. From each part (supernatant and pellet), a small sample (~10 µl, or pipette tip) was taken for testing in SDS-PAGE. The supernatant was supplemented with one tablet protease inhibitor (cOmplete Protease Inhibitor Cocktail Tablets, Roche, Basel, Switzerland) before it was frozen (-20°C), together with the remaining pellet.

To verify the different expression patterns of the AdipoQ protein for each vector, SDS-PAGE analysis with subsequent coomassie staining of the gel was used. A 10% Tris tricine gel was loaded with pellets dissolved in sample buffer (5X) and the corresponding supernatant in sample buffer (5X); all samples were boiled at 95°C for 5 min. The gel runs were performed at 120 V for approximately 2 h. Gels were stained overnight in coomassie; the next day they were destained and analyzed by comparing the band sizes with the molecular weight marker. Based on the different expression patterns of the vectors (given in 2.2.2.), we decided to further continue the AdipoQ expression with the His-tagged protein.

1.3.2. Purification of His-tag adiponectin

For purification, the Protino® Ni-TED Resin (Macherey-Nagel, Düren, Germany) was used. The recombinant polyhistidine-tagged proteins were purified by immobilized metal ion affinity chromatography. The resin was dry silica-based and pre-charged with Ni^{2+} ions. Interaction between the His-tag of the recombinant protein and immobilized Ni^{2+} ions caused the binding of the protein to the resin. Tris-carboxymethyl ethylene diamine (TED) is a strong metal chelator with six binding sites for metal ions. Five binding sites are occupied in the coordination sphere; the remaining coordination site of Ni^{2+} is left to bind the protein (Fig. 19).

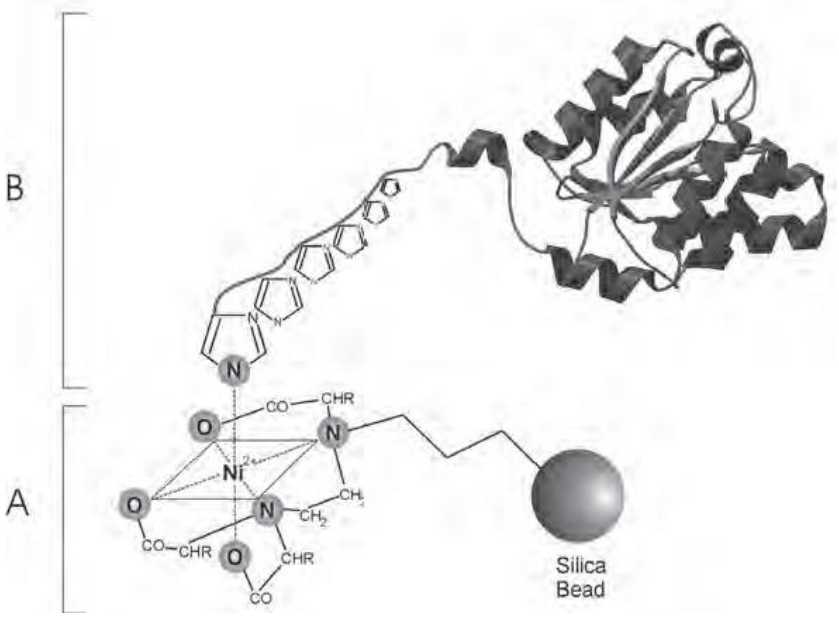


Fig. 19: Binding of a Polyhistidine-tagged protein to Protino® Ni-TED (schematic illustration) A: Protino® Ni-TED, a silica bead, bearing the metal chelator with bound Ni2+ ion. B: One Histidine residue of the Polyhistidine-tag of the recombinant protein binds to the resin.
(MACHEREY-NAGEL – 01 / 2011, Rev. 04)

The purification procedure was carried out as a gravity flow column chromatography under native conditions. Two different buffers were necessary for equilibration, washing and elution (see appendix A).

First, the supernatant was thawed at 4°C and sterile filtered (0.2 µm, Sartorius). Then, 10 mL supernatant were mixed with 1.5 g resin and incubated under shaking for 1.5 h at RT. After incubation, the supernatant resin mix was transferred into a 14 mL column (Macherey–Nagel, Düren, Germany). Before washing, the flow-through was collected. Afterwards, the resin was washed twice with 12 mL LEW buffer; each washing step was collected in separate tubes. Elution was performed in six steps with 6 mL elution buffer used for each step. All eluents were collected in separate tubes. Finally, the resin was washed once with 15 mL LEW buffer to remove residual elution buffer. All buffers and the resin were used more than once; therefore, they were stored at 4°C until further use.

The purity of the eluted protein was verified by SDS-PAGE. For this purpose, a 10% tricine glycine gel was run with samples of all washing and elution steps from one purification procedure. The protein bands were detected by silver staining (Page Silver, Fermentas, St Leon-Rot, Germany).

1.3.3. Concentration and buffer exchange

Buffer exchange was necessary because of the imidazole in the elution buffer, which is not recommended for further use. Vivaspin columns (5 kDa molecular weight cutoff, Sartorius, Göttingen, Germany) were used for buffer exchange and the subsequent concentration of protein solutions. The elution aliquots from one supernatant were pooled and carefully transferred into the Vivaspin columns.

The columns were centrifuged (3000 × *g*, 30 min at 4°C) until 10 mL supernatant were left in the tube; then, the tube was filled up with the elution pool again. This was repeated until the whole elution pool was used and only 10 mL eluent were left in the column. Afterwards, the elution buffer was exchanged by LEW buffer. The 10 mL concentrated eluent in the vivaspin column was filled up with LEW buffer and centrifuged again until 10 mL solution were left. This step was repeated until 100 mL LEW buffer had run through the column and a final volume of 5 mL protein solution was left. Protein concentration was measured by photometer at 280 nm. The extinction was multiplied with the specific AdipoQ extinction factor (1. 0463), which was calculated using Protein calculator v.3.3.

1.4. Endotoxin removal

Protein expression in *E. coli* is associated with endotoxin contamination. Endotoxins consist of lipopolysaccharides (LPS), which are structural components of the outer cell membrane of all gram-negative bacteria. A single *E. coli* cell contains about 2 million LPS molecules (Magalhães et al., 2007). In conditions where the body is exposed to LPS, a systemic inflammatory reaction can occur, leading to multiple pathophysiological effects, such as endotoxin shock, tissue injury, and death (Erridge et al., 2002). However, endotoxin does not act directly against cells or organs but through activation of the immune system, especially through monocytes and macrophages, with the release of a range of proinflammatory mediators, such as tumor necrosis factor (TNF), interleukin (IL)-6 and IL-1. Pyrogenic reactions and shock are induced in mammals and birds upon intravenous injection of endotoxin at low concentrations (1 ng/mL) (Magalhães et al., 2007; Wang et al., 2003).

The purified protein solution was applied to an endotoxin removal resin (Pierce® High- Capacity Endotoxin Removal Spin Column, 0.5 mL, Thermo Fisher Scientific). These columns contain porous cellulose beads, which have been modified at their surface with poly-L-lysine. This modified polylysine has a high affinity to endotoxins.

The procedure was performed according to the manual. Briefly, the endotoxin binding occurrs at pH 6-8, so all buffers were adjusted and the protein was tested further. First, the column was equilibrated to RT and regenerated by 0.2 M NaOH. Afterwards, the column was treated with 2 M NaCl, centrifuged (500 × *g*, 1 min) and followed by a washing step with endotoxin-free water. The last step before the protein solution was loaded, a washing step with endotoxin-free buffer, was repeated three times. Then, the protein solution (3.5 mL) was incubated overnight at RT under shaking conditions (120 rpm). The next day, the endotoxin-free protein solution was collected by centrifugation (500 × *g*, 1 min) of the column and was stored at 4°C until further use.

1.4.1. Limulus Amebocyte Lysate Test

The endotoxin concentration was determined by the Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, USA). Bacterial endotoxin catalyzes the activation of a pro-enzyme in the modified Limulus Amebocyte Lysate (LAL).

The activated pro-enzyme catalyzes the splitting of p-Nitroaniline from a colorless substrate; the activation rate is proportional to the sample endotoxin concentration. After stopping the reaction, the released p-Nitroaniline is photometrically measured (405-410 nm).
The procedure was done according to the instructions of the provided manual. Briefly, preparation of the endotoxin standard stock solutions was performed first. All of the steps were done at 37°C on a heating plate. All standards and samples were pipetted into a 96-well plate and incubated for 5 min. Then, the LAL was added and incubated for exactly 10 min, before the substrate was added and incubated for 6 min. Finally, the reaction was stopped by 25% acetic acid (100 µl per well) and the extinction was measured at 410 nm.

1.5. Application of recombinant adiponectin to test its effects on lymphocyte proliferation

The periparturient period of dairy cows is characterized by a state of immunosuppression, which may result in increased susceptibility towards infectious disease at that time. The immune function and the non-specific host defense mechanisms are altered (Mallard et al., 1998), e.g. functional properties of lymphocytes and monocytes are modulated by parturition and energy balance (Lessard et al., 2004). In particular, the secretion of cytokines like TNFα and INFγ increases after parturition. Furthermore, the proliferative response of PBMC in multiparous dairy cows was lower one week before calving than three weeks before and after calving (Lessard et al., 2004).
We aimed to investigate the effect of AdipoQ on mitogen-stimulated lymphocyte proliferation.

1.5.1. Animals

Blood samples were taken by jugular vein puncture from six multiparous Holstein cows at day 100 ± 7 in lactation. Blood (15 mL) was collected in tubes containing 10 EU heparin/mL blood (Biochrom AG, Berlin, Germany).
The blood sampling was approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (LANUV-NRW, file number 8.87- 51.05.20.10.258, Recklinghausen, Germany). The dairy cows were housed at the experimental research farm of the Faculty of Agriculture, Frankenforst, University of Bonn, Germany.

1.5.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation. The heparinized (10 EU/mL) blood was diluted 1:2 with PBS and carefully layered over lymphocyte separation medium (LSM, PAA, Pasching, Austria).
Afterwards, the cells were centrifuged (1100 × *g*, 30 min, 21°C) without using the break. The PBMCs were harvested from the interface and washed with PBS by centrifugation (430 × *g*, 10 min, 4°C). The supernatant was drained off and the residual erythrocytes were lysed by resuspending the cell pellet in 20 mL cold (4°C) distilled water. The hypotonic lysis was balanced by adding 20 mL 2X PBS. Subsequently, the cells were washed (430 × *g*, 10 min, 4°C). Again, the supernatant was removed and the cells were resuspended in RPMI 1640 medium, supplemented with 100 U Penicillin/mL, 100 mg/mL streptomycin (AppliChem) and 10% FCS (RPMI 1640-S). Further media components are described in appendix A.

1.5.3. Isolation of monocytes and lymphocytes

The affinity of monocytes to bind to plastic was used to isolate monocytes and lymphocytes by adherence. Resuspended PBMCs were layered on Petri dishes (Greiner GmbH, Nürtingen, Germany) and incubated for 1.5 h at 37°C. After incubation, the non-adherent lymphocytes were washed off the Petri dish with pre-warmed (37°C) RPMI 1640 medium and collected in a separate 14 mL Falcon tube (Sarstedt, Nümbrecht, Germany). The monocytes were collected by rinsing with cold (4°C) PBS and by gentle mechanical scraping (Cell scraper, Sarstedt). Both cell types were washed (430 × *g*, 10 min, 4°C) and resuspended in RPMI 1640. Viability and cell count was estimated by Trypan blue and counting in a Neubauer counting chamber. After counting, the cells were finally washed (430 × *g*, 10 min, 4°C) and resuspended in freezing medium. Subsequently the cells were aliquoted into cryo vials (Sarstedt) and directly frozen at -80°C.
The purity of isolated cells was estimated by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg) using forward and side scatter, and that was kindly made available by Prof. Dr. H. Ilges, (Immunology and Cell Biology, Department of Natural Science, Bonn-Rhein-Sieg University of Applied Science)

1.5.4. Isolation of granulocytes

Out of the remaining cells after the PBMC isolation (mainly erythrocytes and granulocytes) by density gradient centrifugation (1.5.2), granulocytes were isolated by repeated hypotonic erythrocytes lysis. First, the plasma and LSM which was left in the centrifugation tube on top of the erythrocyte and granulocyte pellet was discarded. Subsequently, the pellet was diluted in 20 mL cold (4°C) distilled water and inverted several times. The osmotic imbalance was adjusted by adding 20 mL 2X PBS into the falcon tube. Then, the cells were washed (430 × *g*, 10 min, 4°C), the supernatant was drained off and the cells were again resuspended in 20 mL distilled water. The procedure was repeated twice.

Finally, the remaining granulocytes were resuspended in RPMI 1640-S. The viability and cell count was estimated by Trypan blue staining. After final washing (430 × *g*, 10 min, 4°C), the cells were resuspended in freezing medium, aliquoted in cryo vials and directly frozen at -80°C.

The purity of isolated cells was estimated by flow cytometry (FACSCalibur) using forward and side scatter (see above).

1.6. Test protocol for assessing lymphocyte proliferation

The preparation of cells was initiated by warming (up to 37°C) the RPMI 1640-S medium and thawing the lymphocytes. For that purpose, the cryo vials were incubated at 37°C in a water bath, for no more than 1 min, to prevent complete thawing. Afterwards, the lymphocytes were washed (300 × *g*, 8 min, RT) in 20 mL warm (37°C) RPMI 1640-S medium. The supernatant was drained off and the pellet was resuspended in 1 mL warm (37°C) RPMI 1640-S medium. Then, the cells were counted (Neubauer counting chamber) and tested for viability by Trypan blue staining. The desired amount of cells was 1×10^6 lymphocytes per mL and the cells were accordingly diluted and pipetted in 100 µl aliquots into a 96-well plate (Sarstedt); the outer wells, on the left and right side of the plate, were used as blank (only contained RPMI 1640-S medium). Lymphocytes were cultured in quintuplicates, either with or without Concanavalin A (ConA, Sigma-Aldrich, St. Louis, MO, USA) stimulation. The ConA stock solution (1 mg/mL) was thawed and diluted to a final concentration of 10 µg/mL in RPMI 1640-S; 50 µL ConA solution were pipetted into half of the wells on the plate.

The wells in the other half of the plate (unstimulated cells) were adjusted to the same volume with 50 µL RPMI 1640-S medium per well. Each well of the plate contained a volume of 150 µl in which lymphocytes were either stimulated (+ConA) or unstimulated (-ConA).

1.6.1. Preliminary testing the effect of LPS on lymphocyte stimulation

Before testing the effect of recombinant AdipoQ on lymphocyte proliferation, we tested the potential influences of LPS contamination. Concerning the potential LPS contamination of the recombinant AdipoQ solution, we tested the effect of different LPS concentrations on stimulated (+ConA) and non-stimulated (-ConA) lymphocytes. For that purpose, we used pure *E. coli* endotoxin at different concentrations of 0.4 ng/mL up to 100 µg/mL (0.4 ng, 1.6 ng, 10 ng, 100 ng, 1 µg, 10 µg, 100 µg/mL) diluted in RPMI 1640-S. The different final concentrations of LPS were applied to the lymphocytes in 50 µl aliquots.

1.6.2. Testing the effect of adiponectin on lymphocyte proliferation

After preparation of the 96-well plate for the proliferation test, as described in 1.6, the lymphocytes were stimulated with two different AdipoQ concentrations (20 and 60 µg/mL). The AdipoQ stock solution (0.1 mg/mL) was dissolved in RPMI-S and pipetted (50 µL) into each of the wells, excluding the blank wells and the control wells without AdipoQ stimulation, as described in Table 4. In each of the plates, lymphocytes from two individual cows were used and tested in quintuplicate, with or without ConA and with either 0, 20 or 60 µg/mL AdipoQ.

Table 4: Layout for an exemplary 96-well plate of AdipoQ stimulated lymphocyte proliferation test with two individual cows. Blanks denote wells containing only medium, grey highlighted wells are the wells stimulated with ConA, the not highlighted wells are the unstimulated ones, and numbers indicate the AdipoQ concentration in µg/mL

		1	2	3	4	5	6	7	8	9	10	11	12
Cow 1	A	Blank	0	0	0	0	0	0	0	0	0	0	Blank
	B	Blank	20	20	20	20	20	20	20	20	20	20	Blank
	C	Blank	60	60	60	60	60	60	60	60	60	60	Blank
	D	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Cow 2	E	Blank	0	0	0	0	0	0	0	0	0	0	Blank
	F	Blank	20	20	20	20	20	20	20	20	20	20	Blank
	G	Blank	60	60	60	60	60	60	60	60	60	60	Blank
	H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

When the pipetting had been completed, the plate was incubated for 72 h at 37°C and 5% CO_2. Thereafter, the plate was centrifuged (200 × *g*, 5 min, RT) and 100 µl medium supernatant were removed into a new empty 96-well plate and frozen at -80°C. Afterwards, 10 µl Thiazolyl Blue Tetrazolium Bromide (MTT, AppliChem) (5 mg/mL) was pipetted into each well and incubated for a further 4 h at 37°C, 5% CO_2. After 4 h, the lymphocytes were lysed with 100 µl lysis solution per well. Before measuring the extinction, the plate was kept overnight in a drawer at RT to completely dissolve the blue crystals. The next morning, the OD in each well of the plate was determined at 570 nm with a microtiter plate reader (EL800; Bio-Tek Instruments). The calculation of the stimulation index (SI) started by subtracting the mean OD of the blanks from each individual OD measurement. Then the "blank-corrected" mean OD of each qiundruplicate with ConA stiumulation was divided by the "blank-corrected" mean of the respective quindruplicates of the cells without ConA stimnulation.

$$SI = \frac{\text{mean OD of stimulated cells}}{\text{mean OD of unstimulated cells}}$$

1.7. Statistical analysis of the lymphocyte proliferation test

All statistical analyses were done using SPSS 20.0 software (IBM, Ehningen, Germany). The results are displayed as means ± SEM and the level of significance was set at $P \leq 0.05$; a trend was defined as $0.05 > P \leq 0.05$.

The stimulation index data were tested for normal distribution by the Kolmogorov-Smirnov test and for homogeneity of variance by the Levene's test. Normally distributed and homogeneous variances data were tested by the parametric one-way ANOVA test. For group-wise comparison, the post hoc Bonferroni correction was applied.

2. Results and Discussion

2.1. Production of bovine recombinant adiponectin

2.1.1. Amplification of the adiponectin gene

The successful amplification of the AdipoQ gene by PCR was verified in a 1% agarose gel, in which a band with the expected PCR product size of 711 bp (Fig. 20) was obtained. The total sequence of the own sequencing result of bovine AdipoQ gene is available in appendix B.

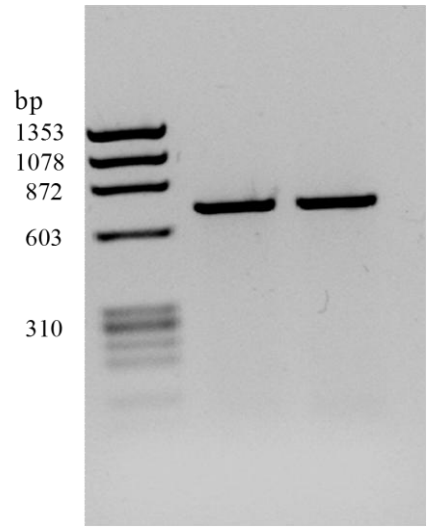


Fig. 20: Electropherogram (1% agarose gel) of the AdipoQ gene after PCR product purification. The sample was loaded in duplicate and size was estimated by comparison with the marker bands

2.1.2. Verification of the donor vector

The correct insertion of AdipoQ DNA into the donor vector was verified by restriction analysis with Hind III and XbaI. The unrestricted plasmid used as a control showed two bands, i.e. the round plasmid DNA with a size of 1830 bp and the coiled DNA, which is much bigger. The duplicate of clone 8 showed a band of 1830 bp, which corresponds to the plasmid and a band at 711 bp which is equivalent to the AdipoQ insert (Fig. 21).

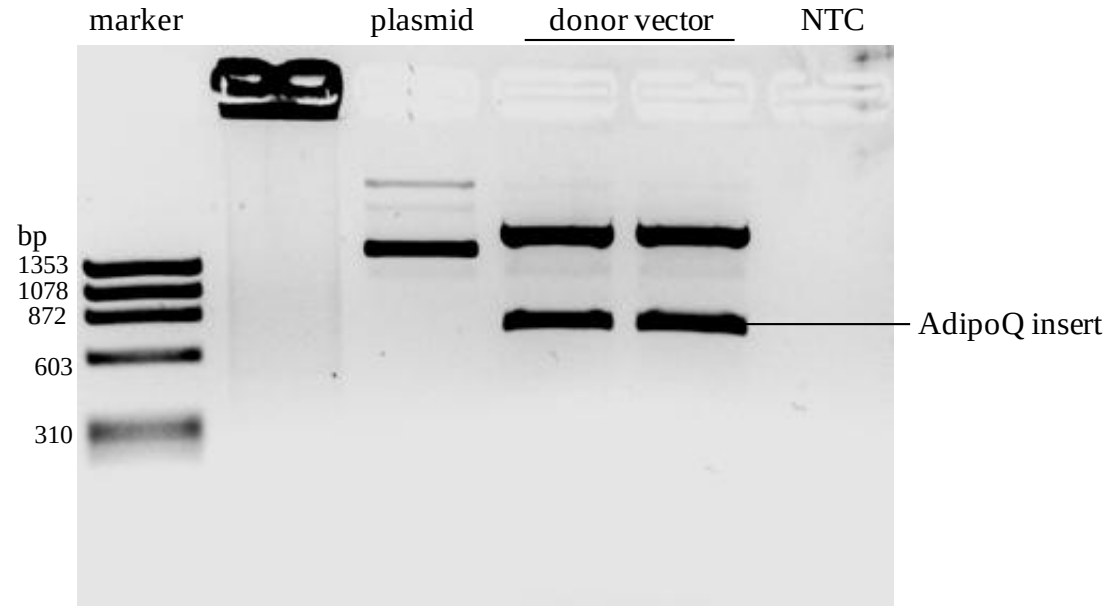


Fig. 21: Electropherogram (1% agarose gel) of the restricted donor vector with Hind III and XbaI. The restriction fragments are shown before (plasmid) and after restriction; the AdipoQ insert showed the expected size of 711 bp. The negative control (NTC) did not show any contamination. Sizes were compared to the loaded marker

2.1.3. Analysis of the destination vector

The precise insertion of AdipoQ gen in the destination vector was analyzed by restriction with HindIII and XbaI. The expected fragments had a length which equated to the AdipoQ insert (711 bp) plus 40 bp of the XbaI and/or HindIII restriction sites and the corresponding tag size. The expected fragment sizes are shown in Table 5.

Table 5: Expected fragment sizes (restriction site + AdipoQ insert + tag size) of cloned destination vectors

Vector	IBA 4 adibos	IBA 35 adibos	IBA 25 adibos	IBA 105 adibos
N-terminal Tag	Strep + signal sequence	6 His	GST	One Strep
restricted fragment sizes [bp]	857	783	1404	855

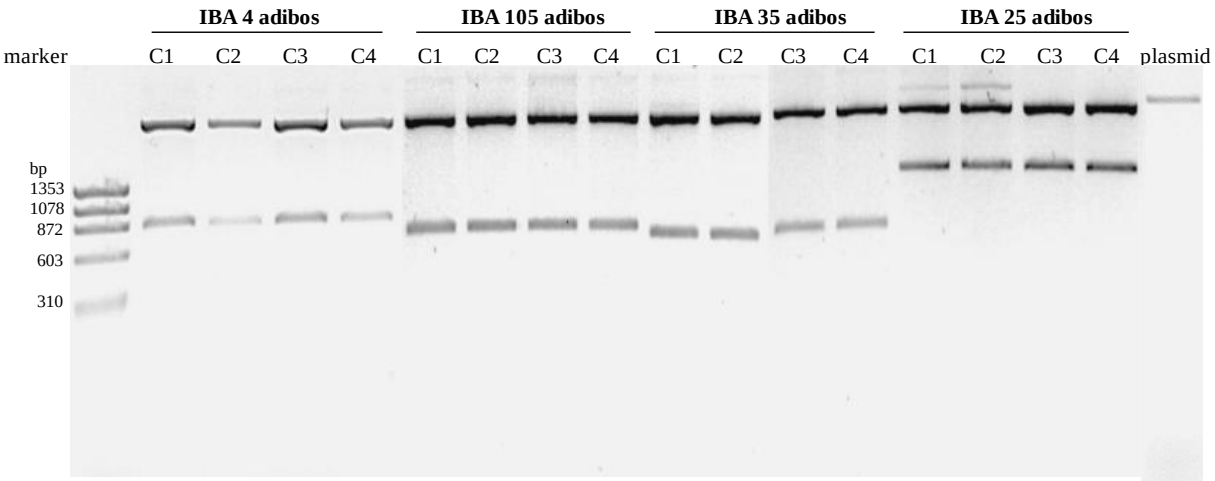


Fig. 22: Restriction analysis of all vectors, size-separated in a 1% agarose gel. Intact plasmid of IBA 4 adibos was used as a control; sizes were compared to the loaded marker

2.2. Overexpression of the adiponectin protein in *E. coli* TOP10 cells

2.2.1. Confirmation of adiponectin expression in different vectors

Within all vectors, protein expression was highest after 4 h of incubation (Fig. 23). The size of the expressed AdipoQ varied according to the specific tag. The different sizes are given in Table 6.

Table 6: Different expected sizes of expressed proteins

Vektor	**IBA 4 adibos**	**IBA 25 adibos**	**IBA 35 adibos**	**IBA 105 adibos**
Tag	Strep	GST	6 His	One Strep
Protein size [kDa]	28	51	26	29

The SDS-PAGE analysis was performed using pellets of the protein expression solution.

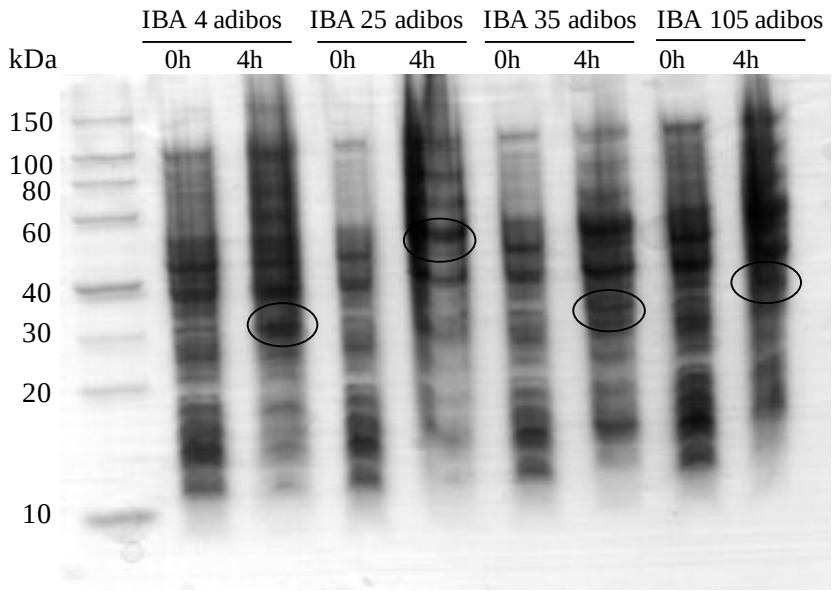


Fig. 23: SDS-PAGE analysis of cell pellets of the overexpressed adiponectin in 4 different vectors. Circles indicate higher protein expression after 4 h at the vector-specific protein size

2.2.2. The appearance of adiponectin in different expression forms

The results presented in Fig. 24 show that AdipoQ was expressed in the soluble form (IBA 25-, 35-, 105 adibos) and in inclusion bodies (IBA 4 adibos). Protein expressed in the soluble form was found in the supernatant. If it was expressed in inclusion bodies, no bands were found in the supernatant. For further AdipoQ expression, IBA 35 adibos was used, since it had most of the expressed protein in the soluble form.

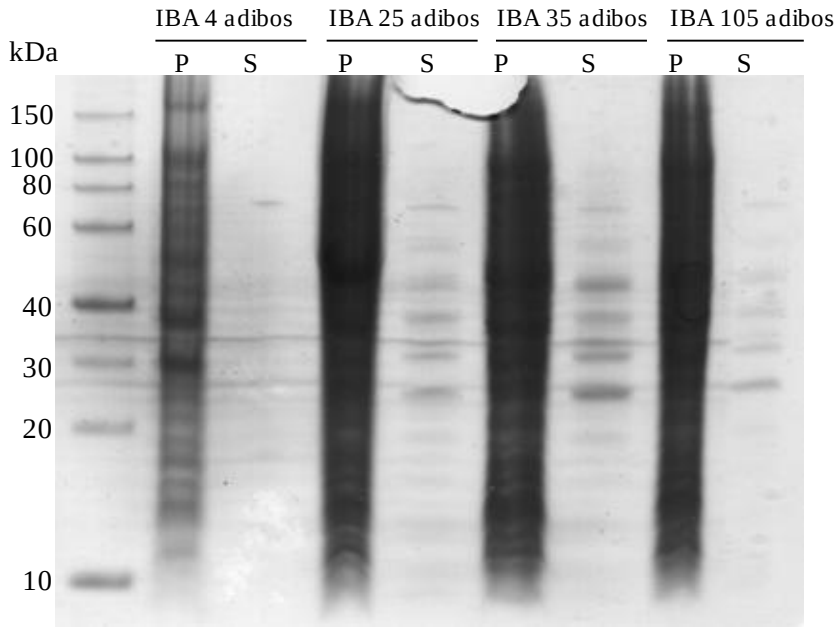


Fig. 24: The expression pattern of AdipoQ in different vectors, verified by SDS Page and coomassie staining. Pellet (P) and the corresponding supernatant (S) from each vector, was loaded on a 12% Tris–Tricine gel

2.3. Adiponectin purification with Ni-TED resin

The sonicated supernatant of His-tagged protein was purified by affinity-binding to the Ni-TED resin. The residual eluents showed bands at approximately 30 kDa and an additional one at around 55 kDa. These bands correspond to the sizes of the AdipoQ monomer and dimer (Fig. 25).

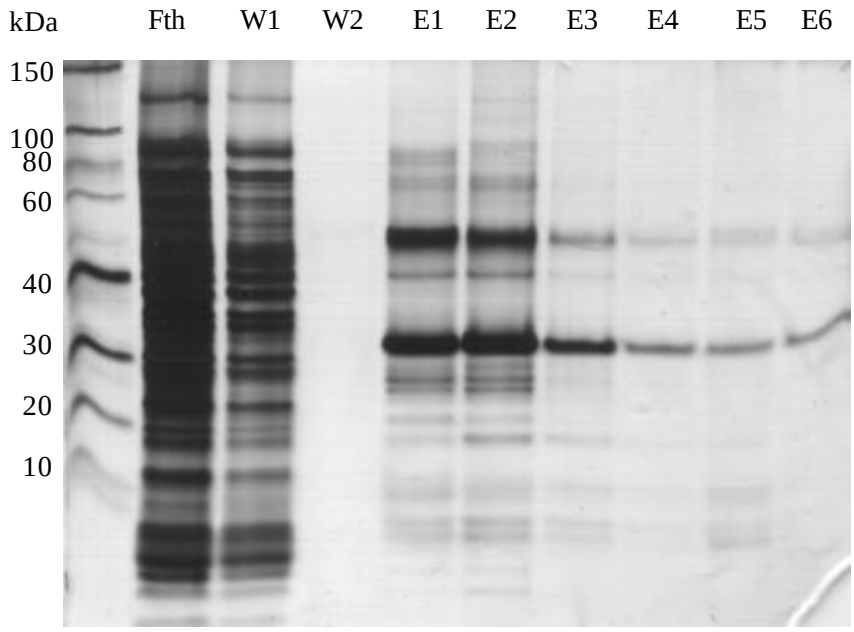


Fig. 25: SDS PAGE of flow through (Fth), washing step 1 and 2 (W1, W2) and 6 elution steps (E1-E6). Protein was non-specifically stained by silver staining. Protein sizes were estimated by comparison to the loaded marker

Furthermore, the collected eluents were applied to Western blot with a specific antibody against His-tags. Herein, all eluents and the washing buffer and flow-through of the purification showed signals for the His-tagged proteins (Fig. 26).

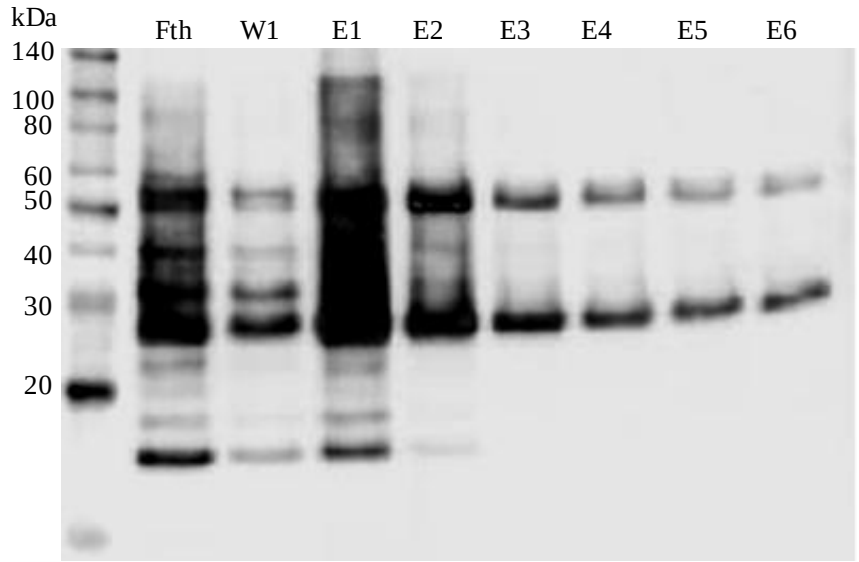


Fig. 26: Western blot detection of purified AdipoQ detected with an anti His-tag antibody. Flow-through (Fth), washing (W1) and eluents (E1- E6).
Eluents with high protein content, i.e. E1- E6, were mixed and concentrated with Viva Spin Columns

The photometrically estimated concentration of protein solution was 0.7 mg/mL. The purified and concentrated protein was verified as bovine AdipoQ by mass spectrometry.

The recombinant AdipoQ was detectable in Western blot with anti AdipoQ antibody (rab14) which was used for all Western blot and ELISA analyses. Recombinant AdipoQ was detected down to a concentration of 250 ng (Fig. 27).

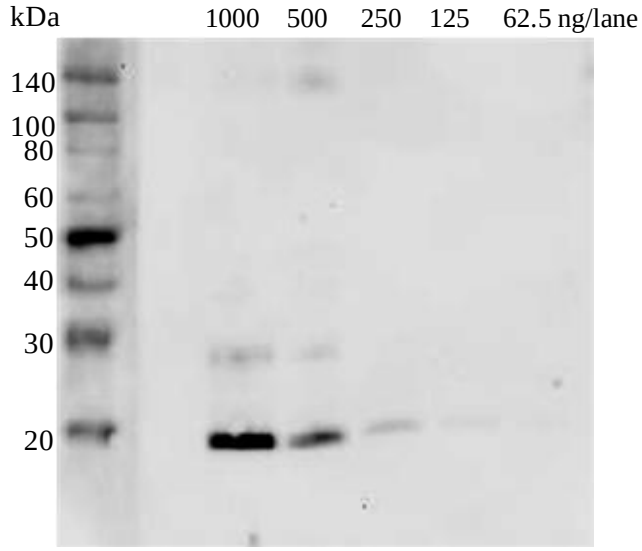


Fig. 27: Dilution series of recombinant AdipoQ analyzed by semi-native Western blot and detected with anti-bovine AdipoQ antibody. Molecular weight of the bands was estimated by comparison to the molecular weight marker

With the use of the Stargate cloning procedure, we were able to express AdipoQ in *E. coli* linked with four different tags. The vector associated with the His-tag showed the highest expression level in the soluble form; therefore, we selected this vector with the His-tag to further proceed with the AdipoQ overexpression. His-tagged proteins are easy to purify from protein solution because of their high affinity to Ni ions. The ion affinity chromatography method used here is a commonly used method for the purification of His-tagged proteins (Waki et al., 2003, Lichty et al., 2005). Because of the small size of the tag (0.8 kDa), it can be used without affecting the biological function of the target protein (Lichty et al., 2005).

One major limitation when expressing eukaryotic proteins in bacteria is their inability to post-translationally modify the expressed protein. In particular, the AdipoQ oligomers need to be extensively modified after translation; hydroxylation and glycosylation are necessary for the intracellular assembly of the oligomeric structures (Wang et al., 2008). Bacterially-generated recombinant AdipoQ lacking posttranslational modifications, such as lysine hydroxylation and glycosylation, can assemble into trimeric and hexameric forms, but not into HMW oligomers (Tsao et al., 2003).

The Western blot detection of eluents with an anti-His-tag antibody yielded two major bands at about 30 and 50 kDa; these bands correspond to the AdipoQ monomer and dimer MW (Waki et al., 2003). Furthermore, the detection of untreated recombinant purified AdipoQ with the rab14 antibody, which was previously used in all Western blot procedures, resulted in two signals: a 30 kDa band and an 18 kDa band. These bands are in line with the AdipoQ monomer (Waki et al., 2003) and with the globular domain of AdipoQ (Waki et al., 2005). Recently, the globular domain of AdipoQ has also been described in purified bovine AdipoQ detected with the same antibody (rab14) (Mielenz et al., 2013).

2.4. Endotoxin contamination

A further challenge of protein expression in bacteria, especially in gram-negative bacteria, is the potential contamination of the protein solution obtained with lipopolysaccharides (LPS). For that reason, we applied our protein solution to an endotoxin removal procedure. However, the commercial LAL test which was used did not yield stable results. Therefore, we repeatedly assessed the concentration (three times) in the protein solution after applying it to the endotoxin removal resin and used the calculated mean of 27 EU/mL. This corresponds to an LPS concentration of 0.5 ng/mL in 20 µg AdipoQ/mL, and 1.6 ng LPS/mL in 60 µg AdipoQ/mL.
The major challenge with LPS measurement in an LAL test is to avoid any contamination by lab instruments (pipette, tip, or 96-well plate). LPS is able to bind to plastic surfaces (Wiuff et al., 2000), which may explain the high variability in the amount of LPS detected in sample. All tubes used for standards and samples were pyrogen free, but the possibility that LPS in the sample binds to the plastic cannot be excluded. However, this would be an advantage for cell culture experiments, since the probability that LPS would interfere with the cells is thereby reduced.
Additionally, the temperature has an impact on performance of the test and the vortexing and mixing procedure of standards may also influence the repeatability of the LAL test. Blechova and Pivdova (2001) tested the LAL test as an alternative for pyrogen tests in rabbits. As negative points of the LAL test, they mentioned that slight disturbances influence the outcome of the test.
Adiponectin is able to bind LPS (Peake et al., 2006). The authors describe the binding of LPS and native AdipoQ (isolated from human serum).

Furthermore, they estimated a bond between HMW recombinant AdipoQ and LPS that was abolished if reduced to trimers and if deglycosylated. The recombinant AdipoQ purified from *E. coli* is not glycosylated (Wang et al., 2004), which indicates that our recombinant bovine AdipoQ will not bind to LPS.

In general, LPS affected lymphocyte proliferation. Activation of immune cells by LPS is mediated by the cell surface protein CD14 (Wright et al., 1990), which is mainly expressed on monocytes, polymorphonuclear leukocytes and on some B-lymphocytes (Heine et al., 2001), as well as the Toll like receptor 4 (TLR4). The TLR4 generates an innate immune response following LPS stimulation (Poltorak et al., 1998). A study showed that human T-cells respond to LPS mediated by TLR4; LPS up-regulated the T-cell adherence to fibronectin and down-regulated the chemotaxis (Zanin-Zhorov et al., 2007). Additionally, T-lymphocytes are activated by LPS, but their activation depends on accessory monocytes providing co-stimulatory signals (Mattern et al., 1998). However, this kind of stimulation is minimized in our *in vitro* tests, because of the separation of monocytes from PMBCs.

For our *in vitro* functional test we mainly focused on lymphocytes, due to the high reactivity of monocytes and granulocytes to LPS stimulation.

2.5. Immunological test

2.5.1. Isolation of peripheral mononuclear cells

Density gradient flow centrifugation lead to a good separation of PBMC from whole blood. Flow cytometric scatter plots showed only monocytes and lymphocytes after centrifugation (Fig. 28).

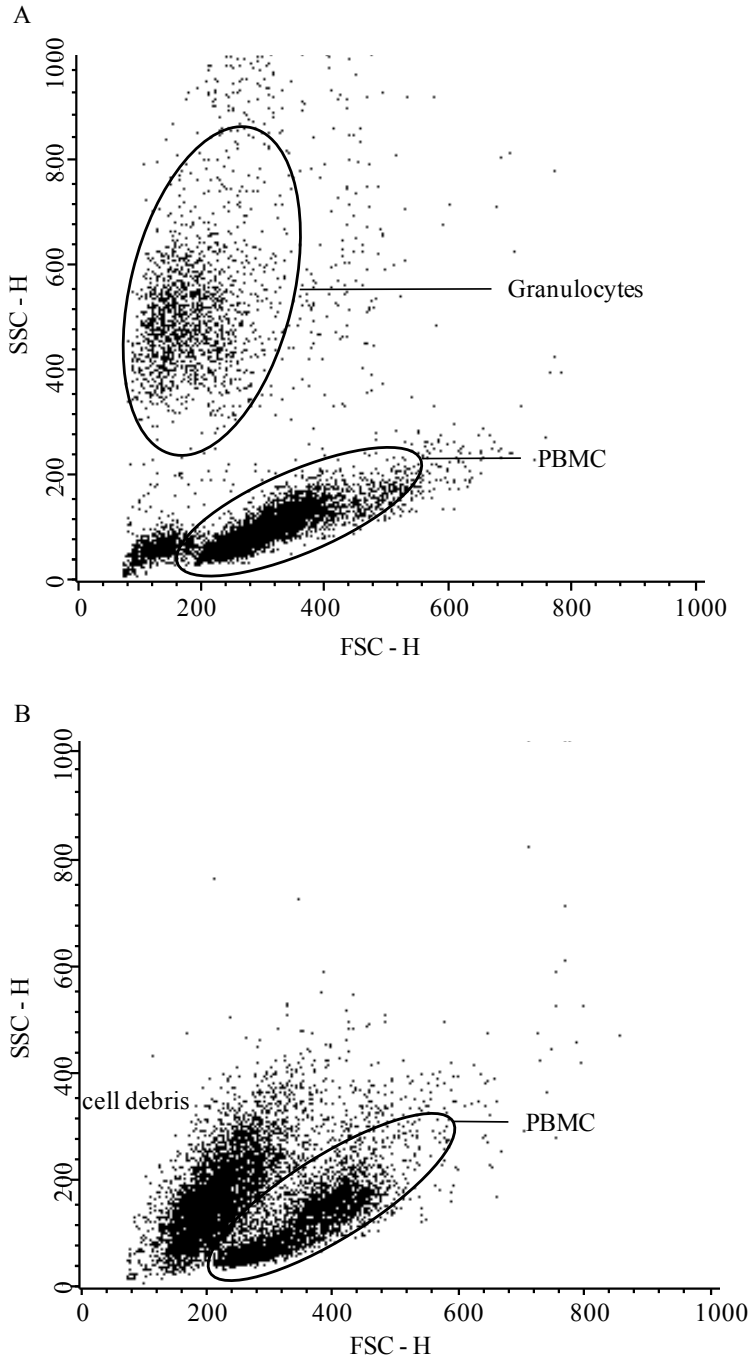
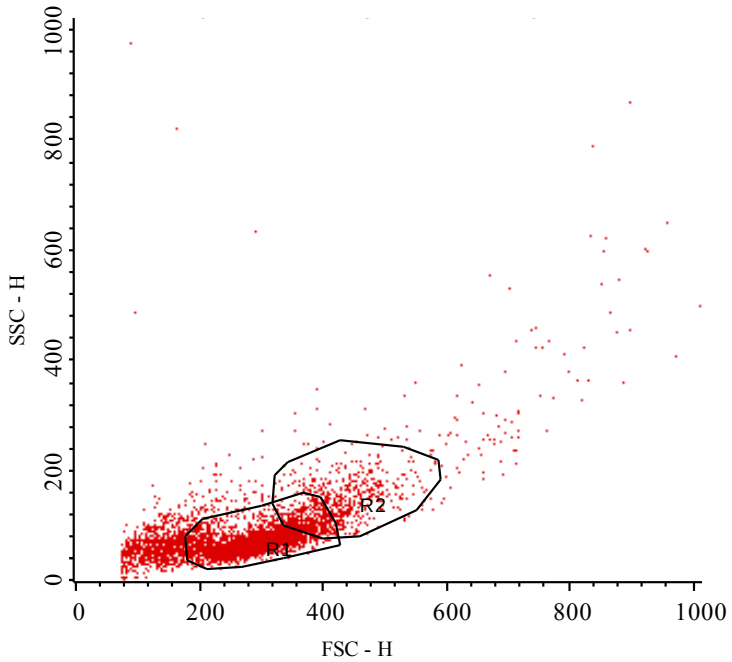


Fig. 28: Scatter plots of whole blood (A) in comparison to PBMCs (B) isolated by density gradient centrifugation (SSC-H: side scatter, FSC-H: forward scatter)

2.5.2. Isolation of monocytes and lymphocytes

The separation of monocytes and lymphocytes by plastic adherence led to 78% pure lymphocytes of all gated cells and 80% monocytes in all gated cells, respectively (Fig. 29).

A



B

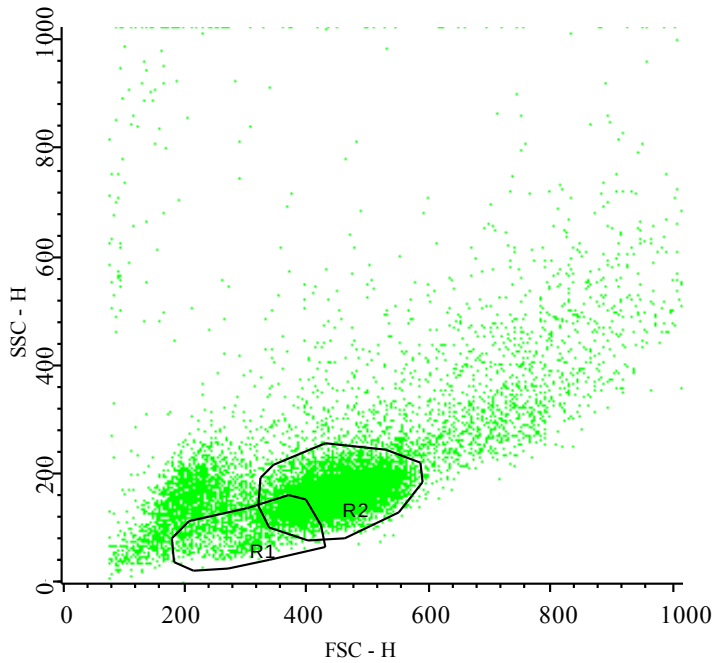


Fig. 29: Scatter plots of isolated lymphocytes (A) and monocytes (B). Cells were gated in relation to their cell size and granularity, R1 is the region for lymphocytes and R2 shows the monocytes. Ungated cells were mainly cell debris or dead cells

2.5.3. Isolation of granulocytes

Granulocytes from the cell pellet after density gradient centrifugation were isolated by repeated erythrocyte lyses. The scatter plot of isolated granulocytes showed a high contamination with monocytes (Fig. 30). In gate R2, 59% of monocytes were gated and in R1 there were still 12% of lymphocytes detected.

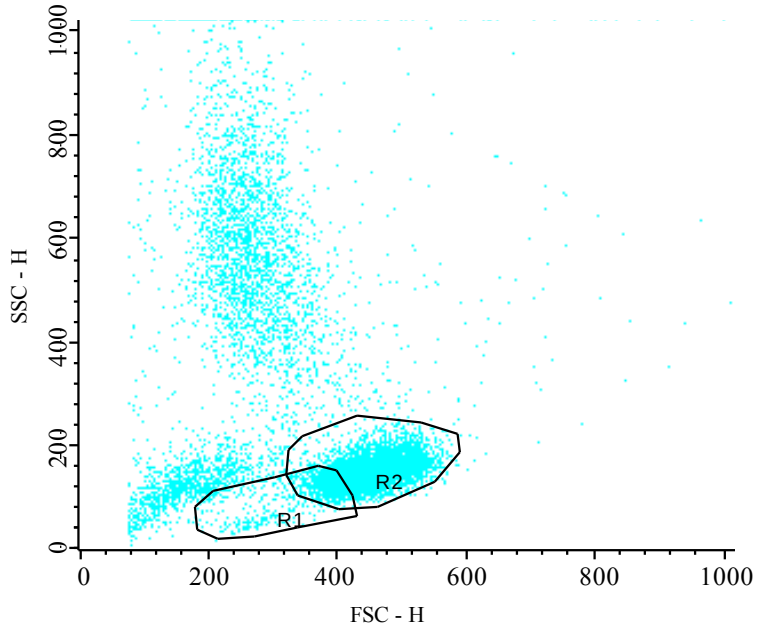


Fig. 30: Scatter plot of isolated granulocytes. Gated cells in R1 were lymphocytes and in R2 monocytes

The separation of monocytes and lymphocytes out of PBMCs by adherence resulted in good purity of the cells. We could not reach the purity of monocytes described in the method that we applied (Dörffel et al., 1999), which might have been partly due to the method used to assess purity. Dörffel et al. (1999) used monocyte-specific antibodies to detect monocytes, while we applied the cell solution to flow cytometry without any specific antibody. Also, the isolation of granulocytes described by Siemsen et al. (2007) differed from ours, which might explain the contamination in isolated granulocytes. After the lysis of erythrocytes, we did not apply the residual cell suspension to a density gradient (Siemsen et al., 2007). As we decided to use lymphocytes for the functional test, we did not continue with the optimization of granulocyte isolation.

2.6. Lymphocyte proliferation test

2.6.1. Influence of lipopolysaccharide (LPS) contamination on lymphocyte proliferation

Lymphocytes stimulated with ConA and LPS at the same time had decreasing stimulation index values with increasing LPS concentrations. The LPS concentrations corresponding to the concentrations estimated in the recombinant AdipoQ preparation decreased the stimulation index. A 20% decrease in stimulation was observed at an LPS concentration of 0.4 ng/mL and with an LPS concentration of 1.6 ng/mL, the stimulation was reduced by 26% in comparison to unstimulated cells.

Lymphocytes treated with LPS in concentrations from 0.4 ng up to 100 µg, but without ConA, showed no differences in proliferation.

2.6.2. Effects of recombinant bovine adiponectin on lymphocyte proliferation

The stimulation with 20 and 60 µg/mL AdipoQ decreased lymphocyte proliferation. At 20 µg/mL, the stimulation index was 61% of AdipoQ-free controls ($P = 0.102$) and 60 µg/mL reduced the stimulation index ($P < 0.05$) to 49% (Fig. 31).

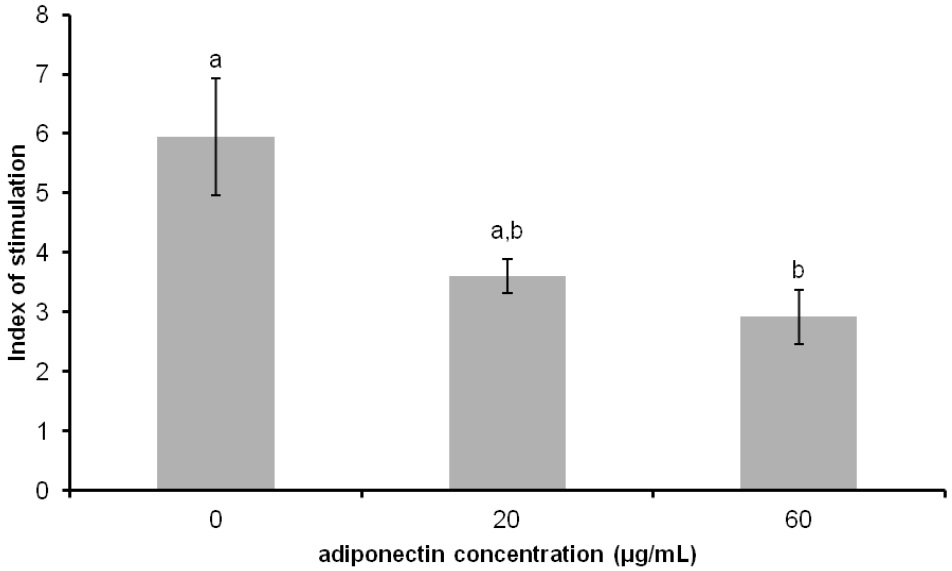


Fig. 31: Stimulation index of lymphocytes incubated with or without 20 or 60 µg/mL recombinant bovine adiponectin (means ± SEM; n=6); different letters indicate significant differences ($P \leq 0.05$).

Decreasing lymphocyte proliferation was observed with increasing AdipoQ concentrations. The AdipoQ concentrations used here mirrored the serum concentration at parturition (20 µg/mL) and a very high, but still physiological concentration (60 µg/mL) observed in individual cows at mid-lactation (Mielenz et al., 2013). Ahmed et al. (2008) observed that leptin, another important adipokine, exerts a dose-dependent suppression of anti-CD3-induced proliferation of bovine T lymphocytes. Both AdipoQ receptors were estimated by flow cytometry on human immune cell surfaces; the highest expression level, at about 90% of the cells, was found on monocytes, whereas only 10% of lymphocyte subpopulations expressed AdipoRs on their cell surface (Wilk et al., 2011). Additionally, the low AdipoR expression at the cell membrane was explained by the finding that T-cells express AdipoRs intracellularly and AdipoRs are translocated to the cell surface upon antigen-specific stimulation (Wilk et al., 2011). The stimulation of a human T-lymphocyte cell line by AdipoQ (10 µM) did not affect its proliferation ability, but a decrease in the secretion of proinflammatory cytokines was detected (Takahashi et al., 2010). These results indicate that AdipoQ might be involved in immune suppression. However, in view of the increasing AdipoQ concentrations and the normalized lymphocyte activity at later stages of lactation, factors other than AdipoQ are probably more important for regulating lymphocyte proliferation. In addition, the lymphocyte prolifertion might also be influcende due to the LPS contained in the recombinant AdipoQ prepration.

CHAPTER VI: General discussion and conclusion

Adiponectin is mainly secreted by adipocytes and occurs in the circulation in different MW forms as a result of several post-translational modifications (Wang et al., 2008). Herein, we demonstrated that AdipoQ occurs in different MW patterns in different body fluids, whereas it seems that the physiological state and concentration in different fluids does not influence the MW secretion pattern of AdipoQ in cattle. The focus of the present discussion is on aspects of AdipoQ secretion which were not previously mentioned in the chapters or manuscript.

The secretion of AdipoQ oligomers is tightly regulated by molecular chaperones in the endoplasmic reticulum, namely ERp44 (an ER protein of 44 kDa) and DsbA-L (a disulfide-bond A oxidoreductase-like protein) and the oxidoreductase Ero1-Lα (ER oxidoreductase 1-Lα) (Wang et al., 2008; Liu et a., 2008). Adiponectin binds to ERp44, which causes the thiol-mediated retention of AdipoQ and inhibits secretion (Wang et al., 2008). Ero1-Lα is ubiquitously expressed and is thought to be the rate-limiting step in disulfide bond formation in most cell types (Cabibbo et al., 2000); it further promotes the release of AdipoQ from ERp44 (Wang et al., 2007). DsbA-L also binds AdipoQ and is involved in regulating the AdipoQ disulfide bond formation. However, it was not able to form AdipoQ multimers when AdipoQ was incubated alone with DsbA-L (Liu et al., 2008).

Not only is the pattern of AdipoQ secretion influenced by chaperones, but also the total amount might be regulated. Treatment of mice with PPARγ agonists lead not only to increased AdipoQ serum concentrations, but also increased the expression of ERp44 and Ero1-Lα in AT, which identifies the two chaperones as possible targets for PPARγ agonist action (Wang et al., 2007). PPARγ expression in AT of dairy cows was lowest at parturition and increased during the course of lactation (Saremi et al., 2014). We found a similar profile of AdipoQ serum concentrations during the first weeks of lactation. PPARγ is a key regulator of insulin sensitivity and adipogenesis (Rosen and MacDougald, 2006) and is related to AdipoQ secretion. A previous study demonstrated that insulin treatment influences the AdipoQ ERp44 interaction; the increase in AdipoQ resulted from a decreasing affinity between AdipoQ and Erp44, which resulted in the direct secretion of AdipoQ by ER as a trimer (Wang et al., 2007).

Once released by adipocytes, the complex distribution of AdipoQ MW forms seems to be stable. Repeated freezing and thawing of human serum samples did not influence the MW distribution (Schraw et al., 2008). We also confirmed findings with bovine serum assayed by semi-quantitative Western blot and ELISA.

Additionally, *in vitro* studies showed that HMW AdipoQ can be spontaneously formed through the aggregation of smaller oligomers (Briggs et al., 2009). These findings might be beneficial in states of decreased HMW AdipoQ, e.g. insulin resistance, in which either abnormalities in the oxidation processes in the ER or decreased numbers of chaperones occur (Briggs et al., 2009).

The pH is another important parameter for AdipoQ stability; it was found that human AdipoQ is very sensitive to pH changes between 6 and 7, whereas the MW forms of mice AdipoQ were unaffected by variations in pH: with decreasing pH, the HMW complexes were degraded to MMW and trimers (Schraw et al., 2008).

The AdipoQ MW patterns might also be influenced by enzyme degradation. Waki et al. (2005) showed via *in vitro* experiments that the cleavage of AdipoQ into fragments (25, 20 and 18 kDa) is mediated by a protease. The enzyme secreted by a human monocyte cell line cleaves AdipoQ into the smallest form of AdipoQ, the globular domain (18 kDa). As globular AdipoQ is known for its different biological activity compared to full length AdipoQ (Waki et al., 2003), it raises the possibility that the activity of AdipoQ might be modulated by the cleavage (Waki et al., 2005).

Human serum samples treated with EDTA had lower amounts of HWM AdipoQ compared to untreated serum. Furthermore, the addition of calcium to human plasma samples increased the formation of HMW AdipoQ complexes (Banga et al., 2008). Likewise, the binding human of AdipoQ to LPS was calcium-dependent, as described by Peak et al. (2006); they reported an increase of AdipoQ binding to LPS by treatment with EDTA and EGTA, whereas the addition of calcium inhibited the binding.

Taken together, many factors are described to affect the MW patterns of AdipoQ, either before or after secretion. Generally, differences in AdipoQ MW patterns might also be related to the different detection systems used in several studies.

In conclusion, further estimation of the AdipoQ MW pattern is reasonable to identify possible factors influencing the expression patterns of AdipoQ. Altogether, the concentration and MW patterns of AdipoQ are important to elucidate the functional role of AdipoQ in livestock, in particular in dairy cows.

Summary

Adiponectin is one of the most abundant adipokines in circulation. It is secreted by adipocytes in three different molecular weight (MW) forms: a trimer with low molecular weight (LMW), a hexamer with middle molecular weight (MMW) and as a multimeric high molecular weight (HMW) form consisting of 12-18 monomers. Adiponectin is negatively correlated with body fat content and is known to be a key regulator of insulin sensitivity and tissue inflammation. Dairy cows undergo various metabolic changes in the time from late pregnancy to early lactation. The energy demand for milk production cannot be covered by feed intake, which results in a negative energy balance. Mobilizing body fat as an energy source may lead to impaired immune function and a higher risk of infectious diseases. The health status of the cow has a strong influence on fertility.

Due to a lack of species-specific assays, estimating AdipoQ concentrations or the MW pattern was impeded; therefore, this thesis aimed (1) to establish a semi-quantitative Western blot to investigate AdipoQ concentrations in the serum and milk of lactating dairy cows, (2) to develop a semi-native Western blot to characterize the AdipoQ MW patterns in several body fluids at different physiological stages of cows and bulls, and (3) to investigate the effect of recombinant AdipoQ on lymphocyte proliferation.

After validation of the semi-quantitative Western blot by establishing the linearity and reproducibility, the AdipoQ serum concentrations throughout lactation were estimated. Blood samples of six multiparous Holstein cows were collected from day -21 to day 252 relative to calving. The serum AdipoQ concentrations decreased towards parturition, reaching a nadir at day -7 and increased after parturition again until relatively constant levels were attained, beginning at day 21. The decreasing AdipoQ serum concentrations around parturition may enhance gluconeogenesis and improve glucose uptake into the mammary gland for milk production.

The semi-quantitative Western blot was also used to characterize AdipoQ concentration in milk samples from three multiparous dairy cows collected from day 5 until day 21 in lactation. The AdipoQ concentration in milk slightly decreased throughout the first three weeks. The concentration at day 5 in lactation was 2-fold higher compared to the concentrations at day 8 and 11 in lactation. Decreasing milk AdipoQ concentration could be explained by the rapid changes of milk composition in the first weeks of lactation.

The semi-native Western blot was also validated by linearity and demonstration of the bands expected after reducing, denaturing treatment.

Afterwards, it was applied for investigation of the AdipoQ MW patterns in serum samples of four lactating Holstein cows (two multiparous and two primiparous). Sampling 1 day post-partum (p.p.) and 105 days p.p. were selected, as AdipoQ serum concentrations on these days were significantly different. The MW patterns showed no differences, either between days or parity. The changes in blood hormones and metabolites thus rather result in altered AdipoQ concentrations, but not in shifts of the MW pattern.

To define the MW pattern of AdipoQ in milk in early lactation, milk samples from three multiparous Holstein dairy cows were used. Milk samples were collected three times a week at the morning milking beginning from day 5 p.p. until day 21 p.p. The AdipoQ MW patterns in mature milk were apparently not different to those seen previously in serum. The given similarity of serum and milk AdipoQ MW patterns might indicate that milk AdipoQ arises from the circulation into the mammary gland.

We estimated the MW pattern and concentration of AdipoQ in cerebrospinal fluid (CSF) and the serum of dairy cows in the transition period. For that purpose, CSF and the corresponding serum samples were collected from ten German Holstein dairy cows at different days in the transition period. The MW patterns of AdipoQ in CSF and serum samples of individual days in the transition period showed no apparent differences. The MMW band was the most prominent one in all cases, whereas the HMW tented to be faint in all samples, independent of whether the sample was serum or CSF. Furthermore, the MW pattern in serum or CSF did not differ for the different stages of the transition period. The transfer of AdipoQ through the blood brain barrier might explain the similarity of the MW pattern found in serum and CSF.

A further aim was to investigate AdipoQ patterns in cattle, other than *Bos taurus*. Blood samples from Yak (*Bos grunniens*), American Bison (*Bison bison*) and blood and milk samples from Water buffalo (*Bubalus bubalis*) were collected. Semi-native Western blot analysis of blood and milk samples yielded signals in all samples. The MW patterns of AdipoQ in Yak, Bison, Water buffalo and buffalo milk were mostly the same. In all samples, a prominent MMW band (~ 130 kDa) and HMW band (> 250 kDa) were detected. In general, the MW pattern was comparable to the pattern seen before in serum and milk of *Bos taurus*. The missing differences in AdipoQ MW pattern within *Bovidae* might indicate that the MMW form of AdipoQ is the most abundant in cattle.

To characterize the potential effects of experimentally-induced mastitis on AdipoQ MW patterns, milk and serum samples of seven mid-lactating cows were estimated. One quarter per udder was experimentally infused with lipopolysaccharides (LPS). For Western blot analysis, serum and milk samples, before and eight hours after LPS challenge, were used.

The distribution of AdipoQ MW complexes did not vary between before and after LPS challenge, neither in serum samples nor in milk samples. It seems that experimentally-induced mastitis with LPS does not change the secretion pattern of AdipoQ, which additionally supports that milk AdipoQ arises from the circulation.

To characterize the MW patterns of AdipoQ in different adipose tissue (AT) depots, homogenates from visceral (mesenterial, omental, retroperitoneal) and subcutaneous (sc.) (withers, sternum, tail head) AT and from the mammary gland were used. For that purpose, AT and mammary gland homogenates from three different days (1, 42 and 105) in lactation were used. Different AT and mammary gland homogenates showed no differences in the MW-pattern of AdipoQ. In each sample, a HMW and a MMW band was detectable, independent of the stage of lactation. Changes in AdipoQ serum concentrations during lactation might not be reflected in changes in the MW patterns of AdipoQ in bovine AT. The similarity of mammary gland AdipoQ MW patterns to serum AdipoQ MW may indicate that AdipoQ from the serum is mainly present in the mammary gland homogenate.

Additionally, the AdipoQ concentration and pattern in allantoic fluid (AF), and maternal blood samples collected at cesarean sections from four Holstein heifers were estimated. The AdipoQ concentrations of cows were well within the expected range and pattern seen for parturition. In AF, only 0.01% of maternal serum concentration was found. The MW pattern of AdipoQ found in AF was mainly HMW. These findings indicate that the AF AdipoQ might be of fetal origin, whereas the maternal serum pattern did not vary in the studied cows previously.

A further study was carried out to characterize AdipoQ in bovine body fluids related to reproduction. For that purpose, we used seminal plasma (SP) from 29 Holstein breeding bulls and follicular fluid (FF) from 14 Simmental heifers. Additionally, we collected blood samples corresponding to the respective reproductive fluid throughout the estrous cycle. The SP AdipoQ concentrations were 180- fold lower compared to serum concentrations. The correlation ($r = 0.843$, $p < 0.01$) between both fluids indicates the origin of SP AdipoQ in circulation. Both fluids had the HMW and the MMW form of AdipoQ, with the HMW form of AdipoQ being more abundant in SP than in serum. Another finding of this study was the age-dependent increase of AdipoQ concentration in serum and SP. Increasing AdipoQ concentrations with age might be due to a reduction in sex hormones, which possibly influence AdipoQ secretion.

The AdipoQ concentrations in FF were 1.6-fold lower than the corresponding serum concentrations and the MW forms were different compared to serum.

Since only MMW AdipoQ was detected in FF, we assume that it is locally produced and does not completely originate from the circulation. In serum samples collected across the estrous cycle, a variation ($P < 0.01$) over time was observed. The lowest serum concentration in estrous was found at day 10, which might be in relation to increasing progesterone concentrations during these days. Another finding of this study was the sexual dimorphism which has been described earlier in human and rodent studies. Bulls had lower AdipoQ serum concentrations compared to heifers, whereas no differences were seen in the MW patterns.

In a further experiment, we investigated the effect of bovine recombinant AdipoQ on lymphocyte proliferation. Adiponectin was expressed as an NH_2-terminal $(his)_6$-tagged fusion protein in *E. coli* Top 10 strain. Blood was collected from six mid-lactating (100 days ± 7 postpartum) Holstein cows by jugular vein puncture. Lymphocytes were isolated by density gradient centrifugation and subsequent separation was performed by adherence on cell culture dishes. Proliferation of lymphocytes was stimulated by the mitogen Concanavalin A (10 µg/mL) and analyzed by the colorimetric MTT (dimethylthiazol) assay. To estimate the mitogen-stimulated proliferation rate, 1×10^6 lymphocytes/mL medium were incubated with two different concentrations of AdipoQ, 20 and 60 µg/mL. Both AdipoQ concentrations decreased lymphocyte proliferation, with the 60µg/ml concentration having a stronger effect than the 20 µg/mL concentration. We concluded that AdipoQ might be involved in immune suppression. However, in view of the increasing AdipoQ concentrations and the normalized lymphocyte activity at later stages of lactation, factors other than AdipoQ are probably more important for regulating lymphocyte proliferation. In addition, a possible influence of LPS out of the recombinant AdipoQ-solution might influence the proliferation of lymphocytes.

The results of this thesis provide AdipoQ profiles in several bovine body fluids. Further estimation of AdipoQ MW pattern is reasonable to identify possible factors influencing the expression patterns of AdipoQ. Altogether, the concentration and MW patterns of AdipoQ are important to elucidate the functional role of AdipoQ in livestock, in particular in dairy cows.

Zusammenfassung

Adiponektin (AdipoQ) ist eines der am häufigsten in der Zirkulation vorkommenden Adipokine. Es beeinflusst verschiedene metabolische Prozesse, trägt zur Verbesserung der Insulinsensitiviät bei und mildert Entzündungen im Gewebe. Die Sekretion erfolgt in drei unterschiedlichen Molekulargewichtsformen (MW): als Trimer in der niedermolekularen Form (LMW), als Hexamer in der mittleren Molekularform (MMW), sowie als multimere hochmolekulare Form (HMW), bestehend aus 12-18 Monomeren. Milchkühe sind in der Zeit der späten Trächtigkeit und frühen Laktation vielen metabolischen Veränderungen ausgesetzt. Die für die Milchproduktion benötigte Energie kann nicht durch die Futteraufnahme gedeckt werden und führt zu einer negativen Energiebilanz. Dies wiederum resultiert in einer Mobilisierung von Körperreserven, welche zu einem erhöhten Risiko für Infektionskrankheiten führen kann und möglicherweise auch die spätere Fortpflanzungsleistung beeinflusst.

Auf Grund fehlender speziesspezifischer Assays waren Untersuchungen zu AdipoQ auf Proteinebene beim Rind nur sehr eingeschränkt möglich. Ziel dieser Arbeit war deshalb (1) die Etablierung eines semi-quantitativen Western Blots zur Bestimmung der AdipoQ-Konzentration in Serum und Milch von Milchkühen im geburtsnahen Zeitraum, (2) die Entwicklung eines semi-nativen Western Blots, zur Charakterisierung unterschiedlicher MW von AdipoQ in verschiedenen Köperflüssigkeiten und Geweben und (3) die Untersuchung möglicher Auswirkungen von rekombinanten AdipoQ auf die Funktionsfähigkeit von Lymphozyten.

Nach der Validierung des semi-quantitativen Western Blots an Hand der Bestimmung der Linearität und Reproduzierbarkeit wurden die AdipoQ-Serumkonzentrationen im geburtsnahen Zeitraum und über die Laktation bestimmt. Dazu wurden Serumproben von sechs multiparen Holstein-Kühen von Tag -21 bis Tag 252 relativ zur Geburt entnommen. Die Serum-AdipoQ-Konzentrationen sanken in Richtung Geburt und wiesen die niedrigsten Konzentrationen am Tag -7 auf. Nach der Geburt stiegen sie wieder an und verblieben ab Tag 21 auf einem relativ konstanten Niveau.

Die verringerten AdipoQ-Konzentrationen bis zur Geburt könnten die Glukoneogenese sowie die Glukoseaufnahme der Milchdrüse zur Milchproduktion verbessern. Ergänzende Analysen wurden an Milchproben von drei multiparen Milchkühen durchgeführt. Hierzu wurden Milchproben von Tag 5 bis Tag 21 der Laktation gesammelt. Die AdipoQ-Konzentration in Milch war am Tag 5 der Laktation doppelt so hoch wie an Tag 8 und Tag 11.

Sinkende AdipoQ-Konzentration in der Milch könnten mit der schnellen Veränderung der Milchzusammensetzung in diesem Zeitraum zusammen hängen.

Der semi-native Western Blot wurde durch Nachweis der Linearität und durch Demonstration der zu erwartenden Banden nach reduzierender und denaturierender Behandlung validiert. Anschließend wurde die Methode für die Bestimmung des AdipoQ-MW-Profils in Serumproben von vier laktierenden Holstein-Kühen (zwei multipare, zwei primipare) verwendet. Auf Grund der signifikanten Unterschiede in der AdipoQ-Serumkonzentration an Tag 1 und Tag 105 post partum (p.p.) wurden diese Tage zur Bestimmung der MW-Profile verwendet. Die MW-Profile zeigten keine Unterschiede zwischen den Tagen nach der Kalbung. Die auftretenden Schwankungen in den Bluthormon- und Metabolitenkonzentrationen resultieren zwar in einer Veränderung der Serumkonzentration, aber beeinflussen offenbar nicht das MW-Profil.

Weiterhin wurde das AdipoQ-MW-Profil in der Milch von drei multiparen Holstein-Kühen ermittelt. Dazu wurden Milchproben bei der morgendlichen Melkung 3-mal wöchentlich von Tag 5 bis Tag 21 in der Laktation gesammelt. Die MW in der Milch waren augenscheinlich nicht unterschiedlich zu den MW im Serum. Die Ähnlichkeit des MW-Profils in Serum und Milch deutet darauf hin, dass das AdipoQ in der Milch aus der Zirkulation stammt.

Außerdem wurde das AdipoQ-MW-Profil und die Konzentration in Cerebrospinalflüssigkeit (CSF) von Kühen in der Transitionsperiode bestimmt. Dazu wurden CSF- und Blutproben von zehn Deutschen Holstein Kühen an unterschiedlichen Tagen der Transitionsperiode gesammelt. Das MW Profil in CSF und Serum zeigte zu unterschiedlichen Zeitpunkten in der Transitionsperiode keine offensichtlichen Unterschiede. Die MMW-Form von AdipoQ war zu allen Zeitpunkten die am häufigsten vorkommende, wohingegen HMW-AdipoQ als schwache Bande zu detektieren war im Serum gleichermaßen wie in CSF. Außerdem hatte auch der Zeitpunkt in der Transitionperiode keine Auswirkungen auf der Verteilung der MW-Formen, weder in den Serum- noch in den CSF-Proben. Adiponektin könnte über die Blut-Hirn-Schranke in das CSF gelangen, was die Ähnlichkeit des MW-Profils zum Serum erklären würde.

Ein weiteres Ziel war die Untersuchg der AdipoQ-Profile in artverwandten Spezies der Rinder (Yak, Bison, Wasserbüffel). Blutproben von Yak (*Bos grunniens*) und amerikanischem Bison (*Bison bison*) und Milch- und Blutproben von Wasserbüffeln (*Bubalus bubalis*) wurden gesammelt. Mittels semi-nativer Western Blot Analyse konnte für alle Proben ein Profil der AdipoQ-MW-Formen erstellt werden. Alle drei Spezies zeigten dasselbe AdipoQ Profil; die MMW Bande war die intensivste und die HMW Bande war bei allen sichtbar.

Generell was das MW-Profil vergleichbar mit dem in Serum und Milch vom Hausrind der Gattung *Bos Taurus.* Die fehlenden Unterschiede in der MW-Verteilung von AdipoQ zwischen den Spezies deuten darauf hin, dass die MMW Form von AdipoQ die am meisten vorkommende im Rind sein könnte.

Um den Einfluss einer experimentell hervorgerufenen Mastitis auf das AdipoQ-MW-Profil zu charakterisieren, wurden Milch- und Serumproben von sieben Kühen in der mittleren Laktation untersucht. In je ein Euterviertel der Kühe wurden Lipopolysaccharide (LPS) infundiert. Zur Analyse im semi-nativen Western Blot wurden Milchproben aus dem behandelten Euterviertel und Serumproben vor und acht Stunden nach der LPS-Infusion verwendet. Die Verteilung der AdipoQ MW-Komplexe zeigte keine Unterschiede vor oder nach der LPS Infusion, weder in Serum noch in Milchproben. Vermutlich wird die AdipoQ-Sekretion nicht durch eine experimentell induzierte Mastitis beeinflusst, was wiederum dafür spricht, dass das in der Milch vorhandene AdipoQ aus dem Blut stammt.

Zur Charakterisierung des AdipoQ-MW-Profils in verschiedenen Fettgeweben (AT) wurden Homogenate aus visceralem (vc.) (mesenterial, omental und retroperitoneal) und subcutanem (sc.) (Widerrist, Brustbein und Schwanzansatz) Fettgewebe und von Eutergewebe verwendet. Zur Analyse im semi-nativen Western Blot wurden Fett- und Euterhomogenate von drei verschiedenen Tagen in der Laktation (1, 42 und 105) verwendet. Es wurden keine Unterschiede in der Verteilung der AdipoQ MW-Formen in den unterschiedlichen Geweben detektiert. In jeder Probe wurde eine HMW- und eine MMW-Bande, unabhängig vom Zeitpunkt in der Laktation, gezeigt. Veränderungen der AdipoQ-Serumkonzentrationen in diesem Zeitraum werden vermutlich nicht im AdipoQ MW-Profil in den verschiedenen AT und dem Eutergewebe wiedergespiegelt. Die Ähnlichkeit des MW-Profils von AdipoQ zum Serumprofil deutet darauf hin, dass das detektierte AdipoQ im Euter aus der Zirkulation stammt.

Des Weiteren wurde die AdipoQ-Konzentration, sowie das MW-Profil in Fruchtwasser (Allantois Flüssigkeit, AF) und dem korrespondierenden maternalen Serum von vier deutschen Holstein Färsen bestimmt. Die AdipoQ-Serumkonzentration der Kühe lag im erwarteten Konzentrationsspektrum zum Zeitpunkt der Geburt. In AF wurden nur 0,01% der maternalen Serumkonzentration gefunden. Das AdipoQ-MW-Profil in AF wies hauptsächlich HMW-Banden auf. Diese Ergebnisse deuten auf einen fetalen Ursprung des in AF gefundenen AdipoQs hin. Die MW-Profile des maternalen Serums entprachen denen der zuvor untersuchten Serumproben der Kühe.

Ein weiterer Bestandteil dieser Arbeit war die Charakterisierung von AdipoQ in bovinen Körperflüssigkeiten in Bezug zu Reproduktion. Hierzu wurde Seminalplasma (SP) von 29 Holsteinzuchtbullen und Follikelflüssigkeit (FF) von 14 Simmental-Färsen gesammelt. Zu jeder Flüssigkeit wurden korrespondierende Serumproben während des Östruszyklus gewonnen. Die AdipoQ-Konzentration der SP Proben zeigten 180- fach niedrigere Werte als die der korrespondieren Serumproben. Die Korrelation der beiden Flüssigkeiten deutet auf einen Transfer vom Blut in SP hin. Beide Flüssigkeiten zeigten HMW- und MMW- AdipoQ- Formen, wobei in SP mehr HMW als in Serum zu finden war. Ein weiteres Ergebnis dieser Untersuchung war ein altersbedingter Anstieg der AdipoQ-Konzentration in Serum und SP. Ansteigende AdipoQ Konzentration im Alter könnten durch die im Alter reduzierten Sexualhormonkonzentrationen erklärt werden.

In der FF war 60% der AdipoQ Konzetration des korrespondierenden Serums zu finden. Auch das MW-Profil zeigte Unterschiede. Dass nur MMW AdipoQ in FF detektiert wurde, lässt vermuten, dass es auch lokal produziert wird und nicht ausschließlich aus der Zirkulation stammt. In Serumproben aus dem Verlauf des Östuszyklus wurden Schwankungen ($P < 0.01$) in der AdipoQ-Konzentration ermittelt. An Tag 10 im Zyklus wurden die niedrigsten AdipoQ-Serumkonzentrationen ermittelt. Sie könnten im Zusammenhang mit steigenden Progesteronkonzentrationen in diesem Zeitraum stehen.

Ein weiteres Ergebnis dieser Untersuchung war ein Sexualdimorphismus der AdipoQ-Serumkonzentration: Bullen hatten niedrigere AdipoQ-Konzentrationen im Vergleich zu Färsen, wohingegen kein Unterschied bei den MW-Profilen gefunden wurde.

In einem weiteren Experiment wurde der Effekt von rekombinantem bovinem AdipoQ auf die Lymphozytenproliferation ermittelt. Dazu wurde Adiponektin als NH_2-Terminales $(his)_6$ markiertes Fusionsprotein in *Escherichia coli* exprimiert. Blutproben zur Gewinnung von Lymphozyten wurden von sechs Holstein-Kühen in der Mitte der Laktation (Tag 100 ± 7) gesammelt. Die Lymphozyten wurden mittels Dichtegradientenzentrifugation und einer anschließenden Plastikadherenz separiert. Die Proliferation der Lymphozyten wurde durch das Mitogen Concanavalin A (Con A, 10 µg/mL) stimuliert und mittels eines kolorimetrischen Tests analysiert. Um die mitogen-stimulierte Proliferationsrate zu bestimmen, wurden 1×10^6 Lymphozyten/mL Medium mit zwei unterschiedlichen AdipoQ-Konzentrationen, 20 und 60µg/mL, inkubiert. Beide AdipoQ-Konzentrationen führten zu einer reduzierten Lymphozytenproliferation, wobei die Proliferation bei 60 µg/mL stärker reduziert war als bei 20 µg/mL.

Dies lässt darauf schließen, dass AdipoQ in Immunsuppressionen involviert sein könnte, wenn man jedoch die steigenden AdipoQ-Konzentrationen und die wieder normalisierte Lymphozytenfunktion im Verlauf der späteren Laktation in Betracht zieht, sind möglicherweise andere Faktoren wichtiger als AdipoQ in der Regulation der Lymphozytenproliferation. Zudem kommt ein möglicher Einfluss von LPS aus der rekombinanten AdipoQ-Präparartion, der auch Einfluss auf die Proliferation der Lyphozyten gehabt haben könnte.

Die Ergebnisse der vorliegenden Arbeit zeigen AdipoQ- MW-Profile in verschiedensten bovinen Körperflüssigkeiten. Die Bestimmung der MW-Profile von AdipoQ wird weiterhin von Nutzen sein, um unterschiedlichste Faktoren, die das AdipoQ-Profil beeinflussen könnten, zu identifizieren. Um die funktionale Rolle von AdipoQ in Nutztieren, vor allem in Milchkühen aufzuklären, ist es weiterhin wichtig, die Konzentration und das MW-Profil von AdipoQ gemeinsam zu bestimmen.

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Appendix A: Buffers, chemicals and solutions

1. Western blot buffer:

Resolving buffer

1.5 M Tris (hydroxymethyl aminomethane)/HCl	(Carl Roth, Karlsruhe, Germany)
0.4% SDS (Sodium dodecyl sulfate)	(Sigma Aldrich, St. Luis MO, USA)
pH 8.8	

Stacking buffer

0.5 M Tris/HCl
0.4% SDS
pH 6.8

Resolving gel (8 %)/ (12 %) (2 Gels)

2.7 mL / 4 mL Acrylamide (30%) (Rotiphorese® Gel A)	(Carl Roth)
1.1 mL / 1.6 mL Bisacrylamide (2%) (Rotiphorese® Gel B)	(Carl Roth)
2.5 mL / 2.5 mL Resolving buffer	
3.7 mL / 1.9 mL destilled water	
The above mixture mixed with:	
45 µL 10% Ammonium persulfate (APS)	(Sigma Aldrich)
5 µL Tetramethylenediamine (TEMED)	(Carl Roth)

Stacking gel (5.6%) (2 Gels)

0.80 mL Acrylamide (30%)
0.53 mL Bisacrylamide (2%)
1.25 mL Stacking buffer
3.42 mL H2O
The above mixture mixed with:
40 µL 10% APS
10 µL TEMED

Sample buffer (5- fold)

0.064 M Tris HCl	
1% SDS	
0.01% bromophenol blue	(Sigma Aldrich)
10% glycerol	
pH 6.8	

Running buffer (10-fold)

25 mM Tris	(Carl Roth)
0.2 M Glycine	
0.1% SDS	

Towbin Blot buffer (1-fold)

25 mM Tris/HCl	
192 mM Glycin	
200 mL Methanol (100 mL/ 10% buffer)	(AppliChem)

TBS buffer (10-fold)

0.05 M Tris/HCl
0.9% NaCl,
pH 7.5

TBS-T (Tris-Buffered Saline-Tween)
0.5 mL (0.05%) Tween®20
1 L TBS
pH 7.3-7.4

TBST with Roti®block
9 mL TBST
1 mL Roti® block

2. Adiponectin cloning buffers

10 x TBE-Buffer

0,89 M Tris-Base	(AppliChem, Darmstadt, Germany)
0,89 M Boric acid	(AppliChem)
0,02 M EDTA (pH 8)	(AppliChem)
ad 1000 mL ultrapure water, autoclaved	

Agarosegel (1 %)

1X TBE Puffer	
0. 75 g Ultrapure Agarose	Life Technologies GmbH, Darmstadt, Germany)
30 µl) Ethidium bromide (1 mg/ mL)	(eurobio, France)
ad 75 mL ultrapure water	

Orange G-Loading buffer DNA-Gels (5x Buffer)

50 mg Orange G	(Chroma Gesellschaft, Münster,Germany)
2 mL Glycerin	(Carl Roth, Karlsruhe, Germany)
400 µL EDTA (0,5 M, pH 8,0)	(AppliChem)
ad 10 mL ultrapure water	

Colony PCR

10 mM dNTPs each	(Thermo Fisher Scientific, Schwerte, Germany)
20 pmol Stargate Primer Adiponectin, HPLC purified	(Sigma Aldrich, St. Luis MO, USA)
2 U/ µL Fusion™ high fidelity DNA polymerase	(Thermo Fisher Scientific)
Puffer polymerase fuion buffer	
µl ultrapure water	

Restriction

HindIII (1 µl/ 1 µg DNA) (10 U/µl)	(Thermo Fisher Scientific)
XhoI (Er0691) (1 µl/ 1 µg DNA) (10 U/µl)	
Tango buffer 2x	
ad 20µl ultrapure water	

3. Adiponectin expression buffer

LB Agar

40 g Agar	(Carl Roth)
ad 500 mL	
autoclave	

LB medium

25 g Agar	(Carl Roth)
ad 1000 mL	
autoclave	
100 mg/L ampicillin	

LEW buffer
50 mM monosodium phosphate (AppliChem)
$NaH_2PO4 \times H_2O$
300 mM sodium chloride (AppliChem)
NaCl
pH 8.0

Elution buffer
50 mM monosodium phosphate (AppliChem)
$NaH_2PO4 \times H_2O$
300 mM sodium chloride (AppliChem)
NaCl
500 mM Imidazole (Carl Roth)
pH 8.0

Coomassie staining
2.5 g Brilliant Blue R-250 (Carl Roth)
450 mL Ethanol (AppliChem)
90 mL glacial acetic acid (Carl Roth)
ad. 1000 mL

Destaining
100 mL Methanol
150 mL glacial acetic acid

4. Immune cell isolation and proliferation test

Freezing medium
500 mL RPMI 1640
100 U / 100 mg/ mL Pen/Strep
40 % FCS
10 % DMSO (Carl Roth)

RPMI 1640-S
500 mL RPMI 1640 (PAA, Austria)
10 % FCS (FCS gold, PAA)
100 U / 100 mg/ mL Pen/Strep (PAA)

Lysis solution
0. 01 M HCL (Carl Roth)
with 10 % SDS

5. Tissue analyses

Homogenisation buffer
10 mM HEPES pH 7.4
complete® protease inhibitor cocktail (Roche, Mannheim, Germany)
1 tablet/10 ml buffer

Appendix B: Adiponectin sequences

1. ***Bos taurus*** **adiponectin**, C1Q and collagen domain containing (AdipoQ), mRNA Adiponectin sequence (NM_174742.2)

CGCCATCGCCTCCTACTTCCACCCTGACTGAAGTCTGTGGCTCTGATTCCACACCTGAGGGGCTCAG
GATGCTGCTGCAGGGAGCTCTTCTACTGCTACTAGCCTTACCCAGTCATGGC**GAGGACAACATGG**
AAGATCCCCCGCTGCCCAAGGGGGCCTGCGCAGGTTGGATGGCAGGCATCCCAGGACATCC
TGGCCACAATGGCACACCAGGCCGTGATGGCAGAGATGGCACTCCTGGAGAGAAGGGAGAG
AAAGGAGATGCAGGTCTTCTTGGTCCTAAGGGTGAGACAGGAGATGTTGGAATGACAGGAGC
TGAAGGGCCACGGGGCTTCCCCGGAACCCCTGGCAGGAAAGGAGAGCCTGGAGAAGCCGCT
TATGTGTATCGCTCAGCGTTCAGTGTGGGGCTGGAGACCCGCGTCACTGTTCCCAATGTACC
CATTCGCTTTACTAAGATCTTCTACAACCAACAGAATCATTATGACGGCAGCACTGGCAAGTT
CTACTGCAACATTCCGGGACTCTACTACTTCTCTTACCACATCACGGTGTACATGAAAGATGT
GAAGGTGAGCCTCTTCAAGAAGGACAAGGCCGTTCTCTTCACCTACGACCAGTATCAGGAAA
AGAATGTGGACCAGGCCTCTGGCTCTGTGCTCCTCCATCTGGAGGTGGGAGACCAAGTCTGG
CTCCAGGTGTACGAGGGTGAAAATCACAATGGGGTCTATGCAGATAATGTCAATGACTCCAC
CTTCACAGGCTTCCTTCTCTACCATAACATTGTTGAATGAATGAGCACCATTAACTCAGAGTCTC
CATTGGGCCAAGCAGCACAAAGTGAAAGAACTACATTGTAGTTAGGAGACCAATTTTATTATCTAG
CTGAGGGGATTCTGAACATCATTCATTTATTCATTCATCAAGTACCTTTTTTAAGAAATGATATACC
ATGTTCCCAGGACAGTCTTGAAGAAGACATGTCCCCTGGCCTCAAGGATCTGTTGTGTAGTGGTAA
TGGAAGGATGATAACATTTACTGGGGAGTTTCATATACATGACAAGATAACTAACTACAAGAAAGT
ATTTGACAGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

The grey part is the signalsequence and the bold one the translated adiponectin nucleotides. In total adiponectin has a size of 669 bp.

2. Amino acid sequence of translated adiponectin:

5'3' Frame

E D N **Met** E D P P L P K G A C A G W **Met** A G I P G H P G H N G T P G R D G R D G T P G E K G E K G
D A G L L G P K G E T G D V G **Met** T G A E G P R G F P G T P G R K G E P G E A A Y V Y R S A F S V
G L E T R V T V P N V P I R F T K I F Y N Q Q N H Y D G S T G K F Y C N I P G L Y Y F S Y H I T V Y
Met K D V K V S L F K K D K A V L F T Y D Q Y Q E K N V D Q A S G S V L L H L E V G D Q V W L Q
V Y E G E N H N G V Y A D N V N D S T F T G F L L Y H N I V E

3. Sequences of the entry vector with the AdipoQ insert

ATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGC
GTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCA
GAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCG
CTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCG
CTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTG
TGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGATTCCAACCC
GGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATG
TAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTG
GTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAC
AAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGAT
CTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAG
GGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCGAGCTTCAGAAGAACTCGTCAAGAAGGC
GATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCC
CATTCGCCGCCAAGCTCCTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCC
ACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAG
CAGGCATCGCCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCTCGCCTTGAGCCTGGCGAAC
AGTTCGGCTGGCGCGAGCCCCTGATGTTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCC
ATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCA
AGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGAT
GACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACG
TCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTTGC
AGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAG
CCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTC
CACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATCCTCGAAG
CATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT
AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCTGGACCCATCTAGCAACCCCTTGGGGCCTCTAAA
CGGGTCTTGAGGGGTTTTTTCTAGAAAAGCGCGTCTCCAATG**A<u>TTGAGGGTCGC</u>GAGGACAACA**
TGGAAGATCCCCCGCTGCCCAAGGGGGCCTGCGCAGGTTGGATGGCAGGCATCCCAGGACA
TCCTGGCCACAATGGCACACCAGGCCGTGATGGCAGAGATGGCACTCCTGGAGAGAAGGGA
GAGAAAGGAGATGCAGGTCTTCTTGGTCCTAAGGGTGAGACAGGAGATGTTGGAATGACAG
GAGCTGAAGGGCCACGGGGCTTCCCCGGAACCCCTGGCAGGAAAGGAGAGCCTGGAGAAGC
CGCTTATGTGTATCGCTCAGCGTTCAGTGTGGGGCTGGAGACCCGCGTCACTGTTCCCAATG
TACCCATTCGCTTTACTAAGATCTTCTACAACCAACAGAATCATTATGACGGCAGCACTGGCA
AGTTCTACTGCAACATTCCGGGACTCTACTACTTCTCTTACCACATCACGGTGTACATGAAAG
ATGTGAAGGTGAGCCTCTTCAAGAAGGACAAGGCCGTTCTCTTCACCTACGACCAGTATCAG
GAAAAGAATGTGGACCAGGCCTCTGGCTCTGTGCTCCTCCATCTGGAGGTGGGAGACCAAGT
CTGGCTCCAGGTGTACGAGGGTGAAAATCACAATGGGGTCTATGCAGATAATGTCAATGACT
CCACCTTCACAGGCTTCCTTCTCTACCATAACATTGTTGAAGGGAGGAGACGCGCTAAAAGCTT
AAAAAAAATGTCGCACAATGTGCGCCATTTTTGTCGAGGGCAATCCAAAGGCGGTAATACGGTTAT
CCACAGAATCAGGGG

Total size 2451 bp. Adiponectin nucelotides are bold (669 bp), the cleavage sites for Hind III und Xba I are highlighted in grey and the cleaveage site for factor Xa (12 bp) is underlined.

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Publications derived from this doctorate thesis

Papers and manuscripts:

1. Mielenz M, Mielenz B, Singh SP, Kopp C, **Heinz J**, Häussler S, Sauerwein H. 2013. Development, validation, and pilot application of a semiquantitative Western blot analysis and an ELISA for bovine adiponectin.Domest. Anim. Endocrinol. 44:121-30.

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3. Singh S.P., **Heinz J**., Dänicke S., Häussler S.and Sauerwein H. 2013. Identification of adiponectin in bovine milk and characterization of its concentrations during early lactation. Book of Abstracts of the 15th International Conference on Production diseases in Farm Animals, 24.-28.06.2013, Uppsala, Sweden, Page 129. ISBN 978-91-576-9150-7