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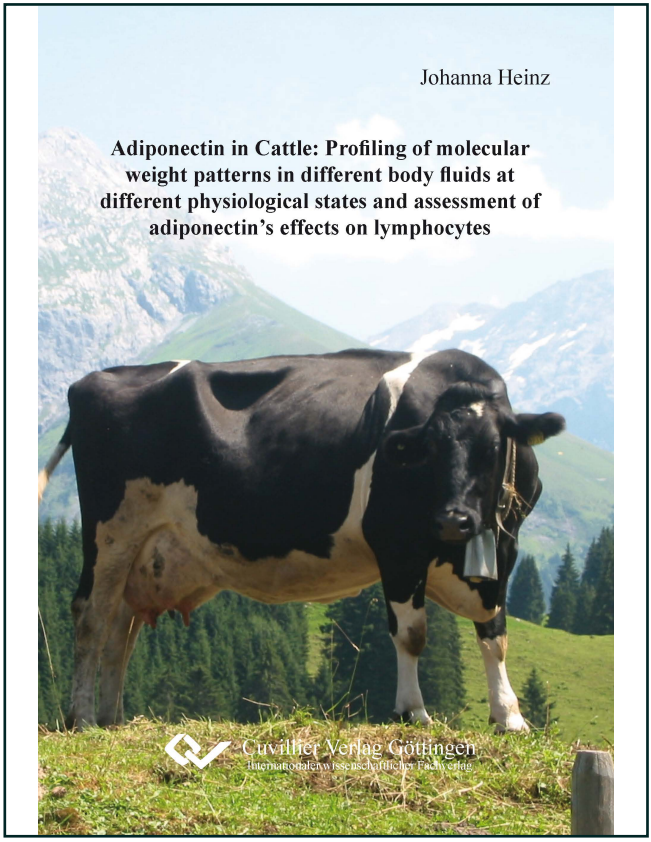
Adiponectin in Cattle: Profiling of molecular weight patterns in different body fluids at different physiological states and assessment of adiponectin's effects on lymphocytes



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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).

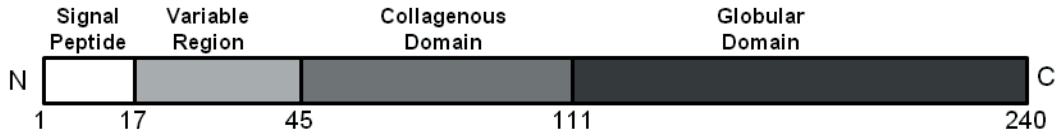

Fig.1: Structural domains of bovine adiponectin. Numbers indicate the first amino acid of the corresponding domain (modified according to Wang et al., 2008)

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).

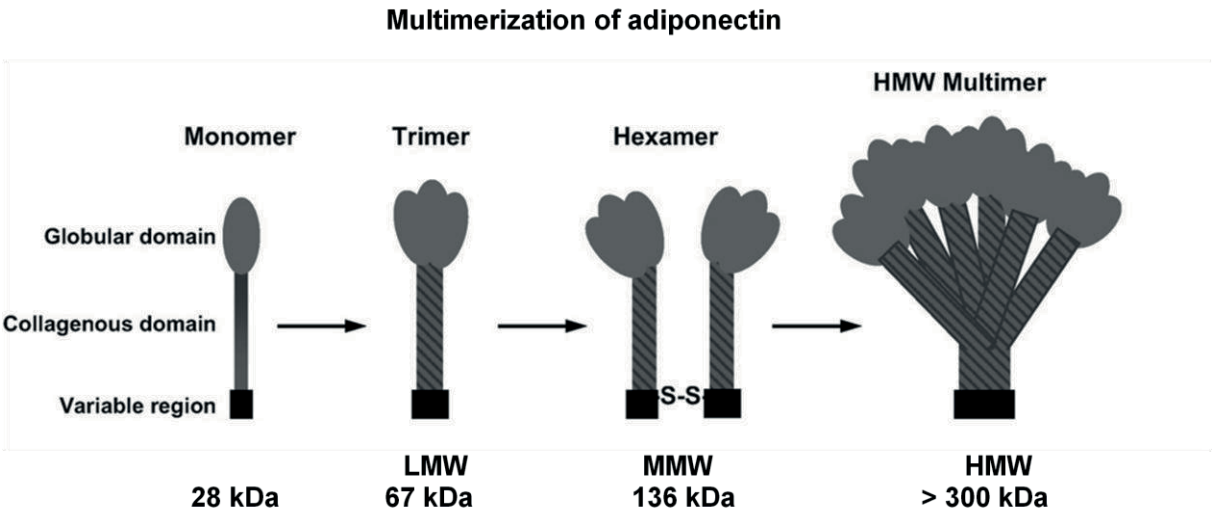

Fig. 2: Multimerization of adiponectin. LMW = low molecular weight, MMW = middle molecular weight, HMW = high molecular weight, and S-S = disulfide bonds (modified from Simpson and Whithead 2010)

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).

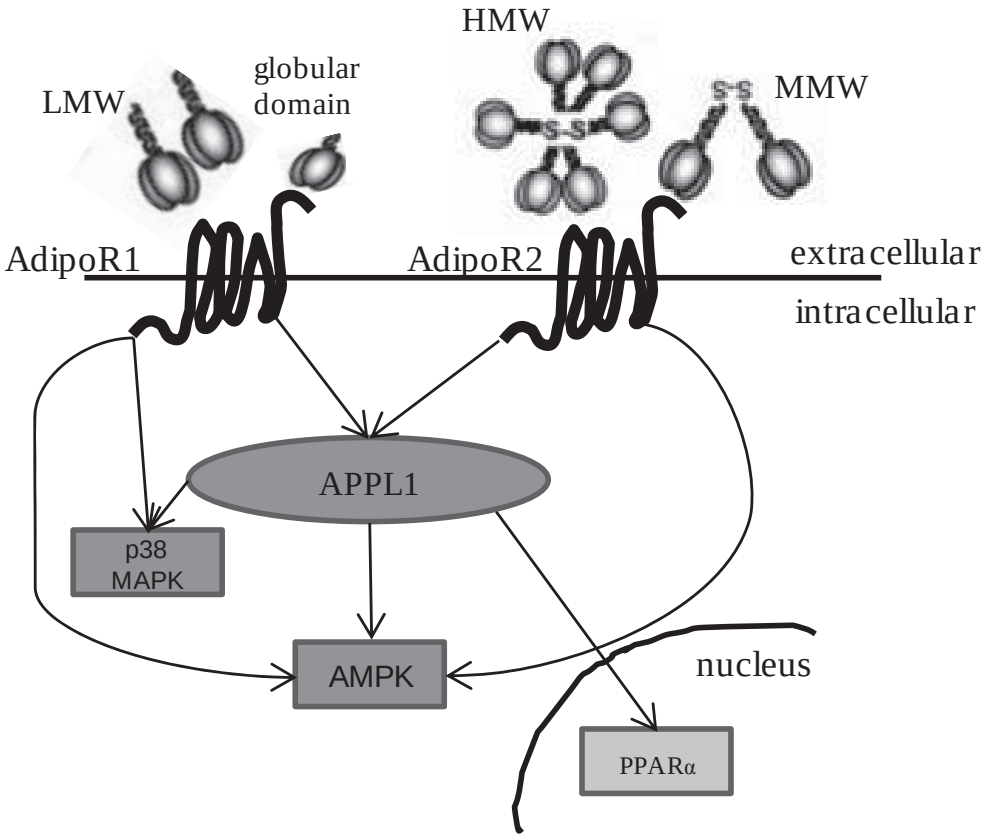

Fig. 3: Adiponectin receptors and signaling. AdipoR1/R2 = adiponectin receptor 1 and 2, APPL1 = adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif, AMPK = adenosine monophosphate-activated protein kinase, PPARα = peroxisome proliferator activated 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