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Effects of magnesium supplementation during the transition period on metabolic profile, immune cell functions and vaccination response in ewes

THESIS

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Dedication

This thesis is dedicated with all my love to the Soul of

My wonderful deeply missed mother Neamat Elmardi

Mona

Parts of the thesis have been previously submitted as manuscripts:

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List of Abbreviations

a.p.	ante-partum
ATP	Adenosine triphosphate
BHB	β-hydroxybutyrate
Ca	Calcium
CD	Cluster of differentiation
cM	Classical monocytes
CNNM2	Cyclin & CBS domain divalent metal cation transport mediator 2
CNS	Central nervous system
ConA	Concanavalin A
CT	Calcitonin
cTAL	cortical segment of the thick ascending loop of Henle
DCT	Distal convoluted tubule
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
ECS	Extracellular space
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GBM	German black headed mutton
GLUT	Glucose transporter
ICS	Intracellular space
IL	Interleukin
intM	Intermediate monocytes
IP3	Inositol trisphosphate
IR	Insulin receptors
K	Potassium
MagT1	Magnesium transporter 1
MAP	Mycobacterium avium paratuberculosis
MCH II	Major histocompatibility complex II molecules
Mg	Magnesium
Mgi	Serum ionized Mg
MgT	Total serum concentrations
mRNA	Messenger RNA

MRS2	Mitochondrial RNA splicing 2
mTOR	mammalian target of rapamycin
Na	Sodium
ncM	Nonclassical monocytes
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NRC	National Research Council
OPT	Ovine pregnancy toxaemia
OVA	Ovalbumin
p.p.	post-partum
PD	Potential difference
PDa	Potential difference of the apical membrane
Pi	Phosphate
PI3K	Phosphatidylinositol-3-kinase
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol 3,4,5-trisphosphate,
PLC ɣ	Phospholipase C- ɣ
PPR	Peri-parturient rise
PT	Pregnancy toxaemia
PTH	Parathyroid hormone
qRT-PCR	quantitative reverse transcription PCR
RNA	Ribonucleic acid
SCFA	Short chain fatty acids
SLC41A1	Solute carrier family 41 member 1
STZ	Streptozotocin
Th1	T helper cell 1
Th2	T helper cell 2
TP	Transition period
TRPM6/7	Transient receptor potential melastatin subtype 6/7

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1 Summary

Mona Hassan Ahmed

Effects of magnesium supplementation during the transition period on metabolic profile, immune cell functions and vaccination response in ewes

High metabolic demands associated with late pregnancy, parturition and the onset of lactation (transition period, TP) are expected to interfere negatively with mineral homeostasis and energy metabolism as well as immune system integrity in farm animals. At cellular level, magnesium (Mg) is a co-factor for more than 600 enzymes involved in energy metabolism, transmembrane fluxes of cations and anions, as well as activation of insulin receptors and glucose transport across cell membranes. Therefore, the present study aimed at investigating whether dietary Mg supplementation has a modulatory effect on the metabolic profile and selected immunological mechanisms during the TP in ewes. To perform this project German black headed mutton breed (GMB) was selected since the mentioned breed is known to be high susceptible to insulin resistance and pregnancy toxaemia during late pregnancy, therefore the selected animals were a good model to investigate the role of Mg supplementation in glucose metabolism during the TP.

For this purpose, 19 pregnant GMB ewes (2^{nd} and 3^{rd} lactation) were divided into control group (n = 9), kept on a ration containing Mg: 0.21% and 0.24% of DM during ante-partum (a.p.) and post-partum (p.p.) periods, respectively, and Mg group (n = 10), supplemented with Mg oxide resulting in a daily Mg intake of approximately 0.30% and 0.38% of DM a.p. and p.p., respectively. Blood was collected at day (d) 30 a.p., d 14 a.p., d 1 p.p., d 14 p.p. and d 30 p.p., respectively. At d 14 a.p., all ewes were vaccinated against *Mycobacterium avium subsp. paratuberculosis* (MAP).

In the first part of the current PhD project the influence of dietary Mg supplementation on mineral (Mg, Ca and P_i), glucose, beta-hydroxybutyrate (BHB) and insulin serum levels were investigated in periparturient ewes. It was revealed that dietary Mg supplementation did not influence the total Mg serum levels. In the control group, total Ca and P_i serum levels were lower at d 1 p.p.. Glucose levels exhibited significant fluctuation only in the control group throughout the TP. Furthermore, during ante-partum period, glucose levels correlated negatively with BHB, and positively with insulin only in control ewes. Lacking such correlations in the Mg group might indicate a modulation of glucose metabolism.

Therefore, it could be speculated that ewes offered more Mg were able to maintain to a considerable extent stable glucose levels throughout the TP compared with the control group, which might decrease the susceptibility of this breed to pregnancy toxemia. Moreover, ewes offered more Mg were able to maintain steady P_i serum levels around parturition and did not show the sharp decrease which was observed in the control group. Parturition is associated with a remarkable alteration in nutrient and metabolic demand, which can be associated with impaired immune function. Therefore, in the second part of the current PhD study, *in vitro* neutrophil phagocytic activity and lymphocyte proliferation as well as *in vivo* vaccination response were investigated in ewes supplemented with Mg during the TP. The results demonstrated that at d 1 p.p. the neutrophil phagocytic activity was higher in the Mg group, furthermore, throughout the TP, the proliferative response of CD4+ cells was higher in the Mg group. Regarding the vaccination response, ewes in both groups responded with an increase in the total blood leukocyte count, neutrophil numbers and non-classical monocytes within 24 h post vaccination, whereas total monocytes and classical monocytes dropped in numbers. Interestingly, numbers of intermediate monocytes only increased in the Mg group, whereas lymphocyte numbers decreased. Mg supplementation did not affect the significant increase in MAP-specific antibodies at d 7 and 21 post vaccination.

In the third part of this PhD project, the gene expression of different glucose transporters (GLUT1, 3 and 4) as well as selective Mg transporters such as SLC41A1, CNNM2, TRPM6, TRPM7, and MagT1 in leukocytes were investigated after two months of dietary Mg supplementation. The results revealed that, higher leukocyte expression of GLUT1 was reported as a tendency in the Mg group compared with the control ewes. Only numerical increases were observed in the expression of GLUT3, SLC41A1, CNNM2, TRPM6, TRPM7, and MagT1 in the ewes offered more Mg. However, GLUT4 was not expressed in either of the two groups.

In conclusion, Ca, P_i and glucose serum levels were more stable throughout the TP with Mg supplementation. This might suggest an approach to use additional Mg during TP to promote metabolic health in GBM ewes. Moreover, neutrophil phagocytic activity, the proliferative capacity of CD4+ cells and the cellular response within 24 h after a vaccination are subject to modulation with dietary Mg supplementation, these findings shed a new light on the role of Mg as an immune modulator, which may thus influence the ewes ‘ability to cope with some pathogens.

For further studies, it would be interesting to determine the serum levels of PTH and $1,25(OH)_2D_3$ since the sharp decrease in serum P_i levels around parturition was only observed in the control group. Furthermore, it is important to investigate the expression of Mg transporter genes in rumen and kidney beside the leukocytes, which might give more information about Mg handling in the body. Moreover, the modulatory effects of Mg on early vaccine/pathogen-induced innate immune responses require further investigation at intracellular signalling pathways level.

2 Zusammenfassung

Mona Hassan Ahmed

Auswirkungen einer Magnesiumergänzung während der Transitphase auf Stoffwechselprofil, Immunzellfunktionen und Impfreaktion bei Mutterschafen

Die hohen Stoffwechselanforderungen im Zusammenhang mit Spätträchtigkeit, Geburt und Beginn der Laktation (Transitphase, TP) stehen im Verdacht, die Mineralhomöostase und den Energiestoffwechsel sowie die Integrität des Immunsystems bei Nutztieren negativ zu beeinflussen. Auf zellulärer Ebene ist Magnesium (Mg) ein Co-Faktor für mehr als 600 biochemische Reaktionen, die am Energiestoffwechsel, an den Transmembranflüssen von Kationen und Anionen sowie an der Aktivierung von Insulinrezeptoren und dem Glukosetransport durch Zellmembranen beteiligt sind. Daher zielte die vorliegende Studie darauf ab zu untersuchen, ob eine Supplementierung mit Mg einen modulierenden Effekt auf das Stoffwechselprofil und ausgewählte immunologische Mechanismen während der TP bei Mutterschafen hat. Für dieses Projekt wurde die Rasse Deutsches schwarzköpfiges Fleischschaf ausgewählt, da diese Rasse bekanntermaßen sehr anfällig für Insulinresistenz und Trächtigkeitsstoxämie ist. Daher sind die ausgewählten Tiere ein gutes Modell, um die Rolle der Mg-Supplementierung im Glukosestoffwechsel während der TPzu untersuchen. Zu diesem Zweck wurden 19 trächtige deutsche schwarzköpfige Fleischschafe (2. und 3. Laktation) in eine Kontrollgruppe (n = 9), die ante partum (ap) und post partum eine Ration mit 0,21% bzw. 0,24% DM Mg erhielt, und eine Mg-Gruppe (n = 10) eingeteilt. Diese wurde mit Mg-Oxid supplementiert, was zu einer täglichen Mg-Aufnahme von ungefähr 0,30% und 0,38% DM a.p. bzw. p.p führte. Blutproben wurden an Tag (d) 30 a.p., d 14 a.p., d 1 p.p., d 14 p.p. bzw. d 30 p.p genommen. An d 14 a.p. wurden alle Mutterschafe gegen *Mycobacterium avium subsp. paratuberculosis* (MAP) geimpft.

Im ersten Teil der PhD-Arbeit wurde der Einfluss der Mg-Supplementierung auf die Mineralstoff- (Mg, Ca und P_i), Glukose-, Beta-Hydroxybutyrat- (BHB) und Insulinserumspiegel bei Mutterschafen in der Peripartalperiode untersucht. Es wurde gezeigt, dass eine Supplementation mit Mg keinen Einfluss auf die Gesamt-Mg-Serumspiegel hatte. In der Kontrollgruppe waren die Gesamt-Ca- und P_i-Serumspiegel an d 1 p.p. niedriger. Die Glucosespiegel zeigten nur in der Kontrollgruppe während der gesamten TP signifikante Schwankungen.

Darüber hinaus korrelierten die Glukosespiegel vor der Geburt nur bei Kontrollmutterschafen negativ mit BHB und positiv mit Insulin. Das Fehlen solcher Korrelationen in der Mg-Gruppe könnte auf eine Modulation des Glucosestoffwechsels hinweisen. Daher könnte spekuliert werden, dass Mutterschafe, denen mehr Mg angeboten wurde, im Vergleich zur Kontrollgruppe wesentlich besser stabile Glukosespiegel im gesamten TP aufrechterhalten konnten, was die Anfälligkeit dieser Rasse für Trächtigkeitstoxämie verringern könnte. Darüber hinaus waren Mutterschafe, denen mehr Mg angeboten wurde, in der Lage, einen konstanten Pi-Serumspiegel um die Geburt herum aufrechtzuerhalten, und sie zeigten nicht den starken Abfall, der in der Kontrollgruppe beobachtet wurde.

Eine Geburt ist mit einer bemerkenswerten Veränderung des Nährstoff- und Stoffwechselbedarfs verbunden, die mit einer Beeinträchtigung der Immunfunktion verbunden sein kann. Daher wurden im zweiten Teil der PhD-Arbeit *in vitro* die Phagozytoseaktivität der Neutrophilen und die Lymphozytenproliferation, sowie *in vivo* die Impfreaktion bei Mutterschafen in beiden Versuchsgruppen untersucht. Die Ergebnisse zeigten, dass bei d 1 p.p. die Phagozytoseaktivität der Neutrophilen in der Mg-Gruppe höher war. Außerdem war in der gesamten TP die proliferative Reaktion von CD4+ -Zellen in der Mg-Gruppe höher. In Bezug auf die Impfreaktion reagierten Mutterschafe in beiden Gruppen mit einem Anstieg der Gesamtblutleukozytenzahl, der Neutrophilenzahlen und der Zahl der nicht-klassischen Monozyten innerhalb von 24 Stunden nach der Impfung, während die Gesamtzahl der Monozyten und der klassischen Monozyten abnahm. Interessanterweise stieg die Anzahl der intermediären Monozyten nur in der Mg-Gruppe an, während die Anzahl der Lymphozyten abnahm. Die Mg-Supplementierung hatte keinen Einfluss auf den signifikanten Anstieg der MAP-spezifischen Antikörper am Tag 7 und 21 nach der Impfung.

Im dritten Teil dieses PhD-Projekts wurde die Genexpression verschiedener Glukosetransporter (GLUT1, 3 und 4), sowie selektiver Mg-Transporter wie SLC41A1, CNNM2, TRPM6, TRPM7 und MagT1 in Leukozyten nach zweimonatiger Mg-Supplementation untersucht. Die Ergebnisse zeigten eine in der Tendenz höhere Expression von GLUT1 in Leukozyten in der Mg-Gruppe im Vergleich zu den Kontrolltieren. Es wurden nur numerische Erhöhungen bei der Expression von GLUT3, SLC41A1, CNNM2, TRPM6, TRPM7 und MagT1 bei den Mg-supplementierten Mutterschafen beobachtet. GLUT4 wurde jedoch in keiner der beiden Gruppen exprimiert.

Zusammenfassend ist festzuhalten, dass die Ca-, P_i- und Glucoseserumspiegel im gesamten TP mit Mg-Supplementierung stabiler waren. Dies könnte einen Ansatz nahelegen, zusätzliches Mg während der TP zu füttern, um die metabolische Gesundheit bei Mutterschafen zu fördern. Darüber hinaus unterliegen die Phagozytoseaktivität von Neutrophilen, die Proliferationskapazität von CD4+ -Zellen und die zelluläre Reaktion innerhalb von 24 Stunden nach einer Impfung einer Modulation durch eine Supplementierung mit Mg. Diese Ergebnisse werfen ein neues Licht auf die Rolle von Mg als Immunmodulator, was somit die Fähigkeit der Mutterschafe, mit einigen Krankheitserregern umzugehen, beeinflussen könnte.

Für weitere Studien wäre es interessant, die Serumspiegel von PTH und 1,25 $(OH)_2D_3$ zu bestimmen, da die starke Abnahme der Serum-Pi-Spiegel um die Geburt nur in der Kontrollgruppe beobachtet wurde. Darüber hinaus ist es wichtig, die Expression von Mg-Transporter-Genen in Pansen und Nieren zu untersuchen, um weitere Informationen über den Umgang mit Mg im Körper zu erhalten. Zusätzlich erfordern die modulatorischen Wirkungen von Mg auf frühe Impfstoff-/Pathogen-induzierte angeborene Immunantworten weitere Untersuchungen auf der Ebene der intrazellulären Signalübertragung.

3 Introduction

3.1 Transition period as a metabolic challenge period

Transition from late pregnancy to the onset of lactation, is one of the most critical periods for farm animals (Transition period, TP). During this time, the energy demand significantly increases to meet the requirements for fetal growth and production of colostrum and milk (Bell, 1995; Yildiz et al., 2005). Consequently, the mother's metabolic and endocrine profiles change dramatically (Ospina et al., 2010; Sordillo et al., 2009). Similar to other farm animals, pregnant ewes undergo metabolic and hormonal changes to meet the fetus's needs during late pregnancy until early lactation (Campion et al., 2016). Minerals such as calcium (Ca), magnesium (Mg) and phosphate (P_i) play an important role in the mineralization of the skeletal system. Hence, as other livestock species, ewes have a high demand for these minerals in late gestation when the fetal skeleton is mineralized.

During lactation, milk production induces high drainage of glucose, amino acids, minerals and other micronutrients from the blood into the mammary gland due to the high concentrations of these compounds in sheep milk compared to other domesticated mammals (Balthazar et al., 2017). Accordingly, during the TP ewes become more susceptible to a wide range of metabolic disorders such as pregnancy toxaemia, hypoglycaemia (Duehlmeier et al., 2013a), hypocalcaemia and hypomagnesaemia (Brozos et al., 2011).

3.2 Minerals and the transition period

3.2.1 Magnesium

Magnesium is an essential mineral and its binding is important for several enzymatic reactions after combining with the enzyme or substrate (Martens et al., 2018). In general, Mg acts as a modulator of synaptic transmission in the central nervous system (CNS) (Morris, 1992; Möykkynen et al., 2001), at the motoric endplate (Lamb and Stephenson, 1994) and in immunological pathways (Li et al., 2011). Furthermore, Mg is involved in the gating of ion channels (Vemana et al., 2008). Most importantly, many transient receptor potential channels are regulated by Mg in a voltage-dependent manner (Voets et al., 2003) and are contributed to the transport of cations across the ruminal epithelium (Leonhard-Marek et al., 2005).

At cellular levels, Mg is the second most abundant intracellular cation with typical concentrations of 10–30 mmol/L. However, since most of the intracellular Mg is bound to

ribosomes, polynucleotides and adenosine triphosphate (ATP), the concentration of ionized Mg falls within the low millimolar range (0.5–1.2 mmol/L) (Ebel and Günther, 1980). Mg is a versatile ion that is involved in practically every major metabolic and biochemical process within the cell. The following paragraphs will highlight the most prominent cellular processes in which Mg is involved:

A. Nucleotide Binding

Mg forms an essential component of the ribonucleic acid (RNA) tertiary structures and stabilization as it binds the negatively charged O and N molecules within the polynucleotide chains (Misra and Draper, 1998). Mg is also crucial to the interactions that stabilize the tertiary RNA structures that are present in messenger RNA (mRNA), ribosomal RNA, transfer-messenger RNA, catalytic self-splicing RNA, and viral genomic RNA (Green et al., 2008).

In DNA, Mg forms hydrogen bonds with the electronegative elements (O, N) to stabilize the natural DNA conformation (Chiu and Dickerson, 2000; Watson and Crick, 1953). Moreover, Mg plays a role in the secondary and tertiary structure of DNA by competing with monovalent ions (Pelletier et al., 1994). In Mg deficient conditions, DNA is more accessible to free oxygen radicals and more prone to oxidative stress (Price and Tullius, 1992).

B. Enzymatic Activity

In medical textbooks and scientific literature, Mg is often described as a cofactor for 300 enzymes. This number has been reported as a rough estimation in 1980 which has been in used since then (Ebel and Günther, 1980). However, in the decades after 1980 many new Mg dependent enzymes have been identified, and the number of 300 is therefore an underestimation. Currently, enzymatic databases list over 600 enzymes for which Mg serves as cofactor, and an additional 200 in which Mg may act as activator (Bairoch, 2000; Caspi et al., 2012). Many of the enzymes that require Mg as coactivator are vital for life (de Baaij et al., 2015).

For instance, Mg is necessary for the structural integrity and activity of DNA and RNA polymerases (Brautigam and Steitz, 1998; Suh et al., 1992). In addition, several enzymes that are involved in protein synthesis and glucose metabolism require Mg (Garfinkel and Garfinkel, 1985; Weber et al., 1967).

Since Mg is involved in many functions, its depletion causes disturbances that may have potentially serious consequences for farm animals, such as depression of voluntary feed

intake, rumen fermentation (Ammerman et al., 1971) and milk production (Wilson, 1980), disturbance of glucose metabolism, minerals imbalance and even death, when hypomagnesemia leads to tetany (Meyer and Scholz, 1972).

3.2.1.1 Mg requirement and homeostasis

Because of the strict relationship between intake of Mg and its plasma concentration, the requirements of ruminants have been established according to the minimum intake required to maintain adequate plasma Mg concentrations (Ammerman and Hemy, 1983).

For instance, net dietary Mg requirements are considered to vary in lactating and nonlactating sheep from 1.2 to 1.8 g/kg feed DMI/day and 0.8 to 1.8 g/kg feed dry matter intake (DMI), respectively (ARC, 1980). Dietary Mg intakes greater than 5 g/kg DMI/day have been observed to result in toxicity in lambs (Chester-Jones et al., 1989).

Around 60–70% of the total body Mg is bound in the skeleton, 25-30% is found in the muscle mass, and only 1% within the extracellular space (ECS) (Martens et al., 2018). The Mg in the ECS only reflects about 1% of total Mg, between 20 and 40% of plasma Mg is bound to albumin and globulin and some 10% complexes with small anions such as citrate, phosphate and bicarbonate, while the rest 50–70% are ionized (free) (Martens et al., 2018). Although the specific mechanisms are not known, plasma Mg is kept within the range between 0.9-1.2 mmol/L, provided that the influx via absorption from the forestomach (rumen and reticulum) into the extracellular space is larger than the efflux into soft tissues and bones for fetal growth during pregnancy, milk production, and intestinal and urinary secretion (Martens et al., 2018). Mobilisation of Mg from bone is unlikely because the ratio between Ca: Mg is 42:1 which would disrupt Ca homeostasis (Fontenot et al., 1989). Therefore, absorption from the forestomachs is probably the key factor determining plasma Mg levels, which can only be kept constant when the daily requirement is adequately balance by ruminal absorption (Martens et al., 2018).

Nevertheless, plasma Mg is known to be influenced in a non-specific manner by catecholamines (Rayssiguier, 1977), insulin (Persson and Luthman, 1974), parathyroid hormone (PTH) (Goff et al., 1986) and epidermal growth factor (Groenestege et al., 2007).

3.2.1.2 Mg absorption from the ruminant gastrointestinal tract

In adult ruminants Mg is mainly absorbed from the forestomachs (Pfeffer and Rahman, 1974). Reduced Mg absorption from the forestomachs could not be compensated by absorption from the intestines (Tomas and Potter, 1976). Mg uptake from the rumen is driven by two luminal mechanisms and one efflux mechanism (Figure 1): Mg uptake (as

an ion through a channel) is driven by the potential difference of the apical membrane (PDa). This mechanism is called potential difference (PD)-dependent or potassium (K)-sensitive Mg uptake, because PDa is mainly modulated by the ruminal K concentration (Leonhard-Marek and Martens, 1996), PD-dependent Mg transport is thought to be mediated by the ruminal epithelial channel such as transient receptor potential melastatin subtype 7 (TRPM7) (Schweigel et al., 2008), and magnesium transporter 1 (MagT1) (Schweigel et al., 2008; Schweigel et al., 2009).

In addition to the channel-mediated pathway, a second, PD-independent/K-insensitive Mg uptake pathway is involved in Mg transport (Leonhard-Marek and Martens, 1996). The charge of Mg is compensated by co-transport with anions or counter-transport of cations. Therefore, the intake of high levels of readily fermentable carbohydrates increased Mg digestion (Giduck and Fontenot, 1987; Martens et al., 1988). Furthermore, Martens et al, (1988) reported that SCFA or CO_2 enhanced ruminal Mg^{2+} absorption *in vivo*. Since both fermentation products acidify the epithelium, $Mg^{2+}/2H^{+}$ exchange has been proposed to represent this transport mechanism (Leonhard-Marek, 1999; Leonhard-Marek et al., 1988). However, a co-transport of Mg^{2+} with an anion such as HCO_3^- or Cl^- is suggested by (Schweigel and Martens, 2003).

Finally, the basolateral extrusion of Mg towards the blood is mediated by an Na^{+} /Mg^{2+} exchanger such as solute carrier family 41 member 1 (SLC41A1) (Kolisek et al., 2012; Schweigel-Roentgen and Kolisek, 2014).

Given that the rumen is the essential site of Mg absorption under various feeding conditions, it has been proposed that both mechanisms work in parallel by ‘job sharing’ with an efficient uptake at all Mg concentrations (Martens et al., 2018). At low ruminal Mg concentrations, the PD-dependent/K-sensitive mechanism mediates Mg transport with high affinity and low capacity (Ram et al., 1998). High ruminal K intake reduced Mg absorption to a higher extent at low ruminal Mg concentration. Consequently, a potential negative effect of K intake will be pronounced at high ruminal K (> 50 mmol/l) and low ruminal Mg (< 2 mmol/l) concentration (Martens et al., 2018).

Vice versa, the PD-independent/K-insensitive mechanism has a high capacity and low affinity and will thus primarily mediate transport at high Mg (> 3 mmol/l) concentrations. This uptake mechanism relies exclusively on the chemical gradients of the involved ions and will rise with increasing Mg concentration (Martens et al., 2018).

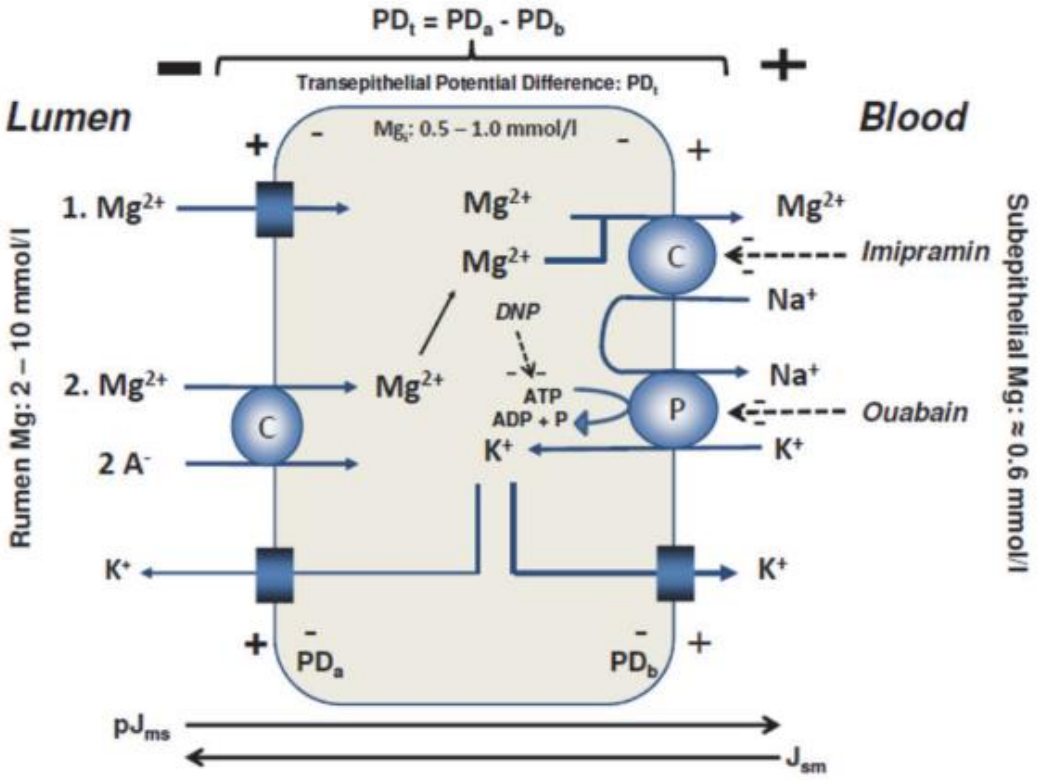

Figure 1. Representation of transepithelial ruminal Mg transport.

The multi-layered epithelium is simplified to one compartment. Passive Mg uptake is driven (1) mainly by the potential difference, PDa, or (2) by the chemical gradient of involved ions. The PD-dependent uptake (1) is thought to be mediated by the epithelial channel TRPM7: transient receptor potential melastatin subtype 7. The molecular identity of PD-independent (2) uptake is unknown. The efflux of the intracellular uptake is mediated via Na/Mg exchange, and the molecular candidate is the SLC41A1 (solute carrier family 41 member 1) Na/Mg exchanger. Mgi: intracellular ionized Mg. The negative effects of inhibitors (-) on various steps of Mg transport are printed in *italics*. pJms and Jsm represent the passive flow through the paracellular pathway. C = carrier; P = Na/K-ATPase (pump). The cylindrical scheme represents a channel. Figure was adapted from (Martens et al., 2018)

3.2.1.3 Factors modulating the ruminal Mg transport

The classical implications of potassium

High dietary potassium (K) intake is a risk factor for hypomagnesaemia as it significantly reduces Mg absorption, plasma Mg concentration and consequently, urinary excretion in sheep (Fontenot et al., 1960). Feedstuffs with K concentration as low as 2 to 3% DM may be tetany prone (Greene et al., 1983). The degree to which high ruminal K antagonises Mg

absorption is dependent on the ruminal Mg concentration (Martens and Schweigel, 2000). At low ruminal Mg levels, the depressive effect of ruminal K levels on Mg absorption is much more pronounced than when rumen Mg concentration is high (Ram et al. 1998; Martens and Schweigel, 2000). Accordingly, dietary Mg supplementation can overcome the negative effect of a high K diet on ruminal Mg absorption (Ram et al., 1998; Martens and Schweigel, 2000).

Sodium Deficiency

Insufficient sodium (Na) intake releases aldosterone and decreases Na in both saliva and rumen fluid, while K is increased which ended by high K:Na ration in the rumen (Martens et al., 1987). These alterations are identical with consequences of high K intake and cause a decrease of Mg absorption.

Protein and ammonia

Dietary protein is converted to ammonia in the rumen by ruminal microbes (Annison et al., 2002), which was shown to reduce the ruminal Mg absorption, independently of K (Care et al., 1984).The reasons of this phenomenon were suggested to be linked to the changes of the ruminal epithelial PD and the pH which interfere with Mg absorption (Martens et al., 2018).

Ruminal pH

Only unbound Mg in rumen fluid is available for transport across the ruminal epithelium (Leonhard et al., 1990). The range of free Mg in the ruminal fluid varies from 34 to 77% of the total amount (Dalley et al., 1996; Grace et al., 1988) which depends on various factors. One major factor determining the solubility of Mg is pH (Dalley et al., 1996). A close negative relationship was reported between rumen pH and Mg absorption (Horn and Smith, 1978). Most likely, increasing pH leads to the deprotonation of anionic binding sites in the ingested matter which are then available for binding of Mg.

Fermentable carbohydrates

A low level of fermentable carbohydrates in tetany-prone grass has been suggested to decrease Mg availability (Metson et al., 1966). In contrast, drenching of grazing dairy cattle with a starch solution increased plasma Mg concentration (Wilson et al., 1969) and digestion of Mg (Giduck and Fontenot, 1987). Moreover, Mg digestion was enhanced in sheep by lactose (Rayssiguier and Poncet, 1980). In ruminal fluid, the addition of fermentable carbohydrates causes: (a) an increase in the concentration of short chain fatty

acids (SCFA) (Giduck et al., 1988), (b) a decrease in pH (Giduck et al., 1988), which (c) enhances Mg solubility (Dalley et al., 1996), (d) a decrease in ammonium concentration, and (e) an increase of the number and size of rumen papilla (Martens et al., 2012), with the latter increasing the area for Mg absorption (Gäbel et al., 1987).

Forages with high water content

A high water content in feedstuff can restrict dry matter intake of ruminants (Clark and Woodward, 2007). A low dry matter concentration in forage may be linked with metabolic diseases such as hypocalcaemia and hypomagnesaemia, by reducing the absorption of key minerals as a result of increased rate of flow of digesta (Foster et al., 2007; Larsen et al., 1986).

3.2.1.4 Renal Mg excretion

There is a close relationship between Mg absorption and renal excretion, (Martens and Schweigel, 2000). Therefore, urinary Mg excretion and Mg concentration in urine are good indicators of whether or not a sufficient amount of available Mg is ingested (Ram et al., 1998).

Plasma Mg varies from 0.9 to 1.2 mmol/L. A total of 60–80% of plasma Mg is ultrafiltrable in the glomerular filtrate (Martens et al., 2018). Most of the ultrafiltrable Mg (about 80%) is reabsorbed, and only 3% to 5% is eliminated in urine (Martens and Schweigel, 2000). Tubular reabsorption of Mg is mainly located in the cortical segment of the thick ascending loop of Henle (cTAL) (60% to 70%), the proximal tubule has a relatively limited role (20% to 25%), the terminal nephron elements including the distal convoluted tubule (DCT) and collecting tubule, reabsorb only a small portion of the filtered Mg (5%) (de Rouffignac and Quamme, 1994; Quamme, 1993).

Moreover, some channels and transports have been reported to be involved in renal Mg handling. For instance, Mg uptake across the luminal membrane in the DCT is mediated by TRPM6 (Houillier, 2014) whose expression is regulated by epidermal growth factor, as it has been considered to be the first autocrine/paracrine magnesiotropic hormone (Groenestege et al., 2007). Moreover, Stuiver et al., (2011) identified a Mg homeostatic protein called protein cyclin and CBS domain divalent metal cation transport mediator 2 (CNNM2), the mutation of this homeostatic protein causes a disturbance in Mg homeostasis. CNNM2 is located in the basolateral membrane of the cTAL and DCT and is upregulated under Mg deficiency condition.

The reabsorption of Mg in cTAL and DCT is closely correlated to plasma Mg concentration. Hypermagnesemia decreases and hypomagnesemia increases Mg absorption in both segments of the nephron (Bapty et al., 1998; Shareghi and Agus, 1982).

3.2.1.5 Effect of adrenaline and noradrenaline on plasma Mg

Transport over long distances, adaptation to sudden outdoor razing, lambing or generally any type of stress lead to an activation the sympathetic system and the hypothalamic–hypophysis–adrenal axis through catecholamine and glucocorticoid production (Miller and O'Callaghan, 2002). Both axis cause a decrease of plasma Mg (Terashima et al., 1983; Yang et al., 1984) which is suggested to be due to a shift of Mg from the ECS into the intracellular space (ICS) (Martens and Schweigel, 2000).

3.2.1.6 Cellular Mg transport mechanisms and/or Mg homeostatic factors

The free intracellular Mg concentration of mammalian cells is held between a relatively narrow range of approximately 0.2 and 1 mmol/L by Mg regulatory mechanisms (Mg transporters/ homeostatic factors) (Nishizawa et al., 2007). Genetic screenings on human diseases and microarray-based expression studies have resulted in the identification of numerous Mg transporting / homeostatic proteins (Figure 2 and Table 1). Major cellular Mg^{2+} influx pathway constituted by: Transient receptor potential melastatin family member 6 and 7 (TRPM6 and TRPM7), and major Mg export mechanism represented by solute carrier family 41 member A1 (SLC41A1), many other proteins were described as being directly or indirectly involved in Mg homeostasis such as magnesium transporter 1 (MagT1) and cyclin and CBS domain divalent metal cation transport mediator 2 (CNNM2), The amount of literature concerning cellular Mg transport and cellular Mg homeostasis is growing, together with a certain amount of uncertainty, especially about the functions (Fleig et al., 2013; Schmitz et al., 2003; Sponder et al., 2016).

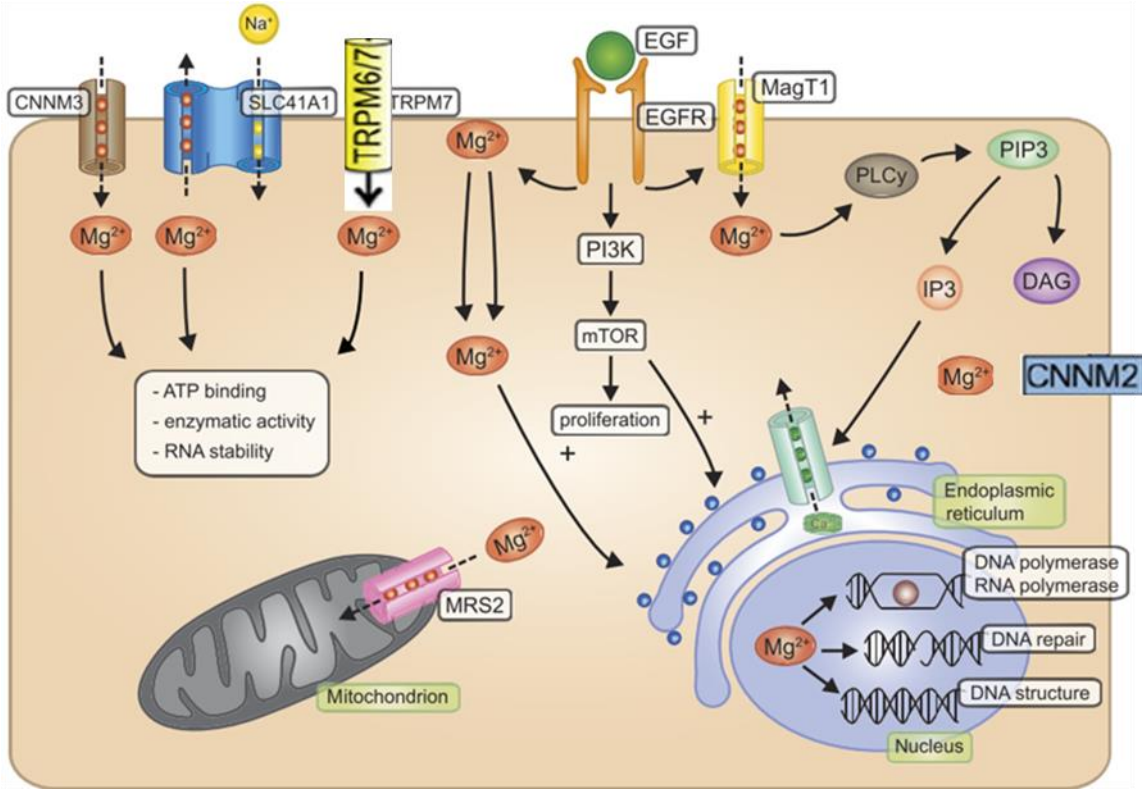

Figure 2. Magnesium transporters.

Cellular Mg homeostasis is regulated by the combined action of TRPM7,6, SLC41A1, MagT1, and CNNM2,3 Mg transporters. MRS2 transporters regulate intramitochondrial Mg concentrations. TRPM7: transient receptor potential melastatin type 6 and 7, CNNM2/3: cyclin and CBS domain divalent metal cation transport mediator 2/3, SLC41A1: solute carrier family 41 type 1, MagT1: magnesium transporter 1; MRS2: mitochondrial RNA splicing 2, EGF: epidermal growth factor, EGFR: epidermal growth factor receptor, PI3K: phosphoinositide 3-kinase, mTOR: mammalian target of rapamycin, PLC γ: phospholipase C- γ, PIP3: phosphatidylinositol 3,4,5-trisphosphate, IP3: inositol trisphosphate. Figure was adapted from (de Baaij et al., 2015) and (Kolisek et al., 2018).

Table 1. Mg transporters, cellular location and tissue expression, table adapted from (de Baaij et al., 2015).

Name	Cellular location	Tissue expression
TRPM7	Plasma membrane	Ubiquitous
MagT1	Plasma membrane	Ubiquitous
SLC41A1	Plasma membrane	Ubiquitous
TRPM6	Apical plasma membrane	Kidney, intestine
CNNM2	Basolateral plasma membrane	Kidney
SLC41A2	Golgi membrane	Ubiquitous
CNNM3	Plasma membrane	Ubiquitous
MRS2	Mitochondrial membrane	Ubiquitous
CNNM1	?	Brain
CNNM4	Basolateral plasma membrane	Intestine

3.2.2 Calcium and Phosphorous requirement and homeostasis

Calcium homeostasis is achieved through the influx of Ca to the blood from intestines, bones and kidneys, and it is under tight hormonal control. In mammals, blood Ca concentration is regulated through a coordinated action of hormones mainly PTH, 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$) and calcitonin (CT) (Horst et al., 1997).

Plasma P_i concentration is regulated by intestinal absorption, bone resorption, urinary and salivary excretion and reabsorption. Hormones involved in Ca regulation also regulate P_i. For instance, 1,25-$(OH)_2D_3$ regulates active absorption of P_i from the intestine, and PTH stimulates salivary and urinary P_i excretion (Goff, 2000).

3.2.3 PTH and 1,25-dihydroxyvitamin D_3

Parathyroid hormone (PTH) is secreted from the parathyroid gland in response to low Ca blood levels. The main function of PTH is to stimulate Ca bone mobilization; additionally it enhances renal tubular reabsorption of Ca, moreover PTH stimulates the kidney to produce 1,25 dihydroxyvitamin D_3 which is required to stimulate intestinal Ca absorption efficiently (Goff, 2000).

P_i absorption in the small intestine is mediated by 1,25-$(OH)_2D_3$, the renal secretion of 1,25-$(OH)_2D_3$ either stimulated directly by low P_i blood levels or indirectly by PTH (Goff, 2000). On the other hand, PTH, secreted during periods of hypocalcaemia, increases renal and salivary excretion of P_i (Goff, 2000).

During late pregnancy and early lactation periods, the requirements of Ca and P_i increase dramatically to meet the needs of the fetus growth and milk production, therefore a significant drop in Ca and P_i blood levels is reported during this time in ewes (Braithwaite, 1983a, b). Thus, an enhancement in the efficiency of above mentioned mechanisms is decisive for the ewes to cope with this mineral imbalance during the TP.

3.2.4 Interaction between Mg and PTH and 1,25-$(OH)_2D_3$

Several studies reported the importance of Mg for PTH secretion and action (Anast et al., 1972; Littledike and Goff, 1987). Additionally, Mg is also essential for renal 1α-hydroxylase activity which is responsible for the production of the biologically active form (1, 25- dihydroxyvitamin D_3) of vitamin D_3 (Rude et al., 2009).

When PTH binds its receptor, activation of adenylate cyclase is initiated, resulting in production of the second messenger, cyclic-AMP, or phospholipase C, which then stimulate the production of additional cellular messengers (Goff, 2000). Both adenylate cyclase and phospholipase C require Mg for full activity. Therefore, hypomagnesemia is capable of interfering with the ability of PTH to act on its target tissues. In humans, it is well recognized that hypomagnesemia can cause hypocalcaemia and that Mg therapy alone restores the serum Ca concentration to normal (Rude, 1998).

In farm animals, subclinical hypomagnesaemia has a negative effect on the release of PTH (Littledike and Goff, 1987; Anast et al., 1972), the functioning of PTH on the target organ (Goff, 2008; MacManus et al., 1971) and the conversion of 25$(OH)D_3$ to 1,25$(OH)_2D_3$ (Horsting and DeLuca, 1969). Furthermore, it was reported that the mobilisation of Ca from bone was lowered significantly in cows with hypomagnesaemia (Sansom et al., 1983).

Therefore, it is suggested that inadequate dietary Mg absorption and hypomagnesaemia are part of the corresponding factors to develop hypocalcaemia in the herd (Goff, 2000).
Regarding P_i homeostasis, an enhancement in P_i plasma levels has been reported recently in periparturient cows supplemented with Mg (Leno et al., 2017). As mentioned above there is no doubt that Mg is essential for Ca and Pi homeostasis.

3.3 Glucose metabolism and insulin function during the transition period

Almost 80% of the fetal growth takes place in the final 6 weeks of pregnancy, with 30–40% of the maternal glucose supply being utilised by the fetal–placental unit (Rook, 2000). Therefore, the higher the number of lambs carried by the ewe, the higher the fetal glucose demands (Sargison et al., 1994). The maternal glucose undersupply is promoted by the fact that placental glucose transport takes place via the insulin independent glucose transporter 1 (GLUT1) while the glucose uptake in the maternal skeletal muscle and adipose tissue is mediated by the insulin dependent GLUT4 (Anderson et al., 2001). Thus, the physiologically impaired insulin sensitivity of ewes during late pregnancy reinforces the disturbance of the maternal glucose supply.
To meet the energy requirements of the growing fetus(es), extensive adaptations of the maternal energy metabolism take place, including increased hepatic gluconeogenesis, reduced glucose uptake by maternal peripheral tissues, and enhanced placental glucose transport capacity (Bell and Bauman, 1997). This “glucose-sparing effect” is at least in part mediated by a physiological peripheral insulin resistance that is exaggerated by undernutrition (Petterson et al., 1993) and probably depends on insulin-desensitizing hormones of gestation and parturition (e.g progestrone and estrogen) (Brănişteanu and Mathieu, 2003). To compensate for the lack of glucose, maternal triglycerides are mobilized, resulting in increased plasma levels of non-esterified fatty acids (NEFA) (Duehlmeier et al., 2013b). Due to the incomplete NEFA break down, the plasma beta-hydroxy-butyrate (BHB) concentrations increase. The elevated BHB levels inhibit the hepatic gluconeogenesis, and thus further increase maternal hypoglycaemia (Schlumbohm and Harmeyer, 2004). Therefore, pregnancy toxaemia (PT) occurs predominantly during late pregnancy in sheep, and not during peak lactation as in dairy cows (Baird, 1981).
Ovine pregnancy toxaemia (OPT) is a disorder of the maternal energy metabolism during late pregnancy, characterized by plasma BHB levels usually higher than 3.0 mM and ketonuria (Wastney et al., 1983).
Several observations, indicated that insufficient energy utilization rather than deficient energy supply is most probably the primary cause of OPT (Marteniuk and Herdt, 1988;

Rook, 2000). One important regulator of nutrient partitioning is insulin (Kahn, 1996). Although glucose utilization in polygastric animals appears to be less responsive to insulin than in monogastric species (Prior and Christenson, 1978) at least an activating effect of insulin on the myocyte glucose uptake was demonstrated in ruminants in vitro (Duhlmeier et al., 2005). Besides its effect on glucose homoeostasis, insulin acts antilipolytic in adipocytes (Wang et al., 2008) and depresses the hepatic ketone body formation (Laffel, 1999). Therefore, insulin resistance promotes impaired glucose supply to the maternal skeletal muscle and adipose tissue, increases lipolysis and enhances ketone body synthesis. As hypo- and hyper-glycaemia, lipaemia and hyperketonaemia are common findings in OPT, insulin resistance may be one major predisposing factor of this affection (Marteniuk and Herdt, 1988).

Thus, it is widely accepted that additional predisposing factors must be involved in the pathogenesis of OPT. As such a breed-dependent factor it has been reported that German black headed mutton (GBM) ewes were recognized as being highly susceptible to OPT (Duehlmeier et al., 2011).

3.3.1 Role of Mg in glucose metabolism and insulin function

Gluconeogenesis is the process of glucose synthesis from noncarbohydrate precursors and Mg is required in three key enzymes involved in this process namely pyruvate carboxylase (Keech and Utter, 1963), phosphoenolpyruvate carboxykinase (Foster et al., 1967) andfructose 1,6-bisphosphatase (McGilvery, 1964). Moreover Mg is involved in the activation of insulin receptors (phosphorylation of tyrosine kinase) and participates in glucose transport across the cell membrane (Goldman and Fisher, 1983).

Insulin receptors (IR) are part of the family of tyrosine kinase receptors, and the kinase function is dependent on the binding of two Mg ions (Hubbard, 1997). Upon activation of the IR, a complex intracellular signalling cascade is activated and mediated via insulin receptor substrate proteins (Taniguchi et al., 2006). In low Mg conditions, activation of the IR may result in diminished signal transduction, contributing to insulin resistance. Studies with hypomagnesemic rats bear this out, as lower IR phosphorylation was detected, although differences between individual organs were reported (Reis et al., 2000; Suarez et al., 1995).

In humans, hypomagnesemia may contribute to the development of diabetes mellitus type 2 by increasing insulin resistance as patients with diabetes mellitus type 2 often have low serum Mg levels (Barbagallo and Dominguez, 2007; Volpe, 2008).

Several studies indicated a beneficial effect of Mg supplementation on reducing plasma fasting glucose levels in patients with type 2 diabetes and on reversing insulin resistance in hypomagnesaemia, non-diabetic and apparently healthy subjects (Song et al., 2006). Furthermore, a previous study provide significant evidence that oral Mg supplementation improves insulin sensitivity even in normomagnesaemic, non-diabetic subjects emphasizing the need for an early optimization of Mg status to prevent insulin resistance and subsequently type 2 diabetes (Mooren et al., 2011).

In a study in rats with streptozotocin (STZ)-induced diabetes, oral Mg supplementation increased GLUT4 expression in the rat muscle and thereby lowered serum glucose levels to the normal range (Solaimani et al., 2014). In farm animals, hypomagnesaemia induced by feeding a low Mg/high K diet reduced insulin responsiveness and insulin-mediated glucose disposal (Matsunobu et al., 1990) and stimulated lipolysis in sheep (Sano et al., 1999). In contrast, ewes supplemented with Mg during the transition period showed a higher efficiency in energy regulation as observed by a decreased in plasma NEFA concentration (Ataollahi, 2018), similar findings were obtained in periparturient cows (Leno et al., 2017).

Several mechanisms may be responsible for the beneficial effect of Mg on insulin resistance. These include direct effects of Mg on the insulin receptor and its downstream signalling processes, additionally, enhances enzyme activities involved in glucose utilization (Mooren et al., 2011).

For this reason, it can either be hypothesized that insulin action is already compromised in the state of marginal Mg deficiency, which cannot be detected with conventional parameters for Mg status, or it can be speculated that Mg in excess may act as a natural insulin sensitizer even under conditions of a well-balanced Mg status (Mooren et al., 2011).

3.3.2 Insulin as a regulatory hormone of Mg metabolism

Insulin has specific ionic effects to stimulate the transport of Mg from the extracellular to the intracellular compartment, thus decreasing serum ionized Mg (Mgi) level (Barbagallo et al., 1993). The insulin-mediated intracellular ionized Mg accumulation may depend upon the activation of the tyrosine-kinase insulin receptor, since the insulin effect on intracellular Mg is totally abolished by a monoclonal antibody directed towards the insulin receptor (Hwang et al., 1993).

Persson and Luthmann, (1974) infused insulin intravenously in sheep and observed a significant decline of plasma Mg after 30 minutes, moreover, an insulin response to infusion of glucose in sheep also caused a decrease in plasma Mg (Yang et al., 1984).

Total serum concentrations (MgT) do not reflect the Mg status or intracellular pool, and intracellular (Mgi) or serum ionized (Mg-ion) Mg depletion can be seen with normal MgT concentrations (Resnick et al., 1993).

3.4 Alterations of immune system during the transition period

Immune dysregulation occurs commonly during the TP, this being due to hormonal fluctuations (e.g. progesterone, cortisol), oxidative stress, negative energy balance, mineral and micronutrients imbalances (Aleri et al., 2016; Lacasse et al., 2018). Changes in immune mechanisms during the TP were reported mainly in cows and to some extent in ewes, such as a reduction in phagocytic activity of neutrophils and macrophages, alteration in the composition of circulating monocyte subsets, production of cytokines, complement activation, proliferation of lymphocytes and the production of antibodies (Eger et al., 2015; Preisler et al., 2000a; Preisler et al., 2000b). Due to these changes, cows show greater susceptibility to a wide range of diseases such as mastitis and retained placenta, while ewes become more vulnerable to gastrointestinal nematodes (peri-parturient rise (PPR) in faecal egg counts) (Beasley et al., 2010).

3.4.1 Hormonal changes and immune dysregulation during the transition period

In reproductive ewes, the most pronounced activation of homeostatic mechanisms in support of multiple pregnancy and lactogenesis, is related to the harmony between the intense hormonal secretions by placenta, anterior pituitary, and multiple corpora lutea (Manalu and Sumaryadi, 1998), which may interfere with the maintenance of physiological equilibrium and the integrity of immunological functions.

Well known that successful pregnancy in mammals depends on maternal recognition and acceptance of the semi-allogeneic fetus and to fulfill this aim the mother's immune system has to be modulated during this time. This modulation consists of a local response (specialized features of the placenta) and a peripheral response (modulation of maternal T cell function) (Chaouat et al., 2004; Veenstra van Nieuwenhoven et al., 2003; Zenclussen, 2005). A downregulation of T helper cell 1 (Th1) cytokines (IFN- α and IL-2) (Wegmann et al., 1993), combined with a bias towards T helper cell 2 (Th2) cytokines (IL-4 and IL-10) was a common feature of mammalian pregnancy (Raghupathy, 1997).

Moreover, during pregnancy, progesterone has often been shown to inhibit many leukocyte functions (Clemens et al., 1979), a phenomenon necessary to prevent rejection of the fetus (Weinberg, 1987).

The act of parturition is a 'stressful event' that induces the production of glucocorticoids accompanied by signalling and coordination from the hypothalamus, pituitary gland and adrenal glands. Effector molecules produced during stress events include norepinephrine, epinephrine and glucocorticoids. Circulating norepinephrine and epinephrine stimulates the production of anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and IL-10 which in turn inhibits the production of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-12. Inhibition of pro-inflammatory cytokine production causes immunosuppression, selectively suppressing cellular immunity and promoting antibody-mediated immunity (Elenkov and Chrousos, 2002; Kasprowicz et al., 2000):

Circulating cortisol levels influence immune responsiveness by directly inhibiting T-cell proliferation, T-cell development, modifying the action of complement molecules and interfering with immunoglobulin function (Lewis, 1997; Mallard et al., 1997). Studies have also demonstrated that circulating cortisol induce down regulation of L-selectin and CD18 expression on the surface of neutrophils, reducing their surveillance activity and as a result, reducing immune response capacity (Burton et al., 2001; Burton et al., 1995; Mallard et al., 2009).

3.4.2 Nutrients imbalance and immune dysregulation during the transition period

The nutritional status of the animals has been associated with the ability to resist to infections. Studies have shown a depression in the blood levels of Ca, Zn, Mg, P_i, K, Se, vitamins A and E during the periparturient period (Goff and Stabel, 1990; Johnston and Chew, 1984; Meglia et al., 2001; Weiss et al., 1990).

Moreover, the onset of lactation induces a negative energy balance and a shift of glucose distribution toward the udder, which may impair the nutrient supply of immune cells. Since they use glucose as an energy source (Pithon-Curi et al., 2004) and their energy demands increase dramatically in terms of activation when they switch from oxidative phosphorylation to glycolysis (Frauwirth et al., 2002).

In periparturient cows, glucose uptake of all monocyte subsets decreased after parturition, moreover glucose transporter genes expression was altered as well (Eger, 2016; Eger et al., 2015).

In ewes, subclinical pregnancy toxaemia during late pregnant was associated with immunosuppression, and concentrations of plasma NEFA and BHB were negatively correlated with immune responses (Lacetera et al., 2001).

3.4.3 Impaired leukocyte activity during the transition period

Neutrophils are one of the key cells in the innate immunity, and their count is a common hallmark for inflammatory response. Neutrophils function primarily to phagocytose and destroy pathogens. Prior to phagocytosis, neutrophils must sense and migrate to the sites of infection via interactions with adhesion molecules and chemoattractant molecules expressed on endothelial linings (Aleri et al., 2016). Once at the site, neutrophils not only phagocytose foreign bodies but are also able to sense and acquire fragments from damaged and dead cells (Whale et al., 2006; Whale and Griebel, 2009). Impaired neutrophilic activity is characterized by reduced activation, chemotaxis, adherence, pathogen ingestion, respiratory burst and release of lytic enzymes (Kimura et al., 2002; Rinaldi et al., 2008; Sordillo and Aitken, 2009). Impaired neutrophil observed in cows during the periparturient period is thought to be primarily due to the effects of glucocorticoids (Preisler et al., 2000a; Preisler et al., 2000b).

Monocytes are immune cells linking innate and adaptive immunity as precursors for tissue macrophages and dendritic cells (Hume et al., 2002). They are functionally characterized by their ability to sense pathogens, to phagocytose microbes, to produce cytokines and chemokines, and to present antigens to T cells using major histocompatibility complex II molecules (MCH II) (Geissmann et al., 2010).

Changes in monocyte subset composition have been shown to influence disease susceptibility and pregnancy outcome in species such as mice, humans and cows (Al-ofi et al., 2012; Melgert et al., 2012; Pomeroy et al., 2016; Tang et al., 2015).

On the other hand, lymphocytes are the key cells in the adaptive immunity as they play a critical role in cell and antibody-mediated immune responses. Reduced numbers of circulating lymphocyte subsets, were reported during the pre-partum period compared with the post-partum period in cows (Harp et al., 1991; Kimura et al., 1999). In sheep, the DNA synthesis of lymphocytes stimulated with Concanavalin A (ConA) was lower around lambing when compared to pre-partum and post-partum (Lacetera et al., 2004) .

A physiological evidence of decreased IgG1 and IgM serum concentrations was observed in the periparturient period in cows (Herr et al., 2011). It is well documented that significant amounts of IgG1 of serum origin are transported into mammary secretions during colostrogenesis (Hurley and Theil, 2011).

Data regarding the vaccination response during the TP are scarce in sheep, a previous study reported that pregnant ewes vaccinated with chicken egg albumin (Ovalbumin, OVA)

showed lower OVA- specific IgG titers compared to the nonpregnant ewes in 4 weeks after the booster dose (Wattegedera et al., 2008).

On the other hand, another study demonstrated that ewes immunized with keyhole limpet hemocyin antigen during the TP showed higher IgG concentrations in single-bearing ewes than twin-bearing ewes before parturition (Caroprese et al., 2006). Additionally the cited authors evaluated the cell-mediated immune status by measuring the skinfold thickness at the side of phytohemagglutinin injection (average value was collected between 24 h postinjection thickness – preinjection thickness), and they reported that the lowest average values were recorded around parturition time (7 days before and after parturition) throughout the whole TP. The immediate cellular response was not addressed in these experiments.

3.4.4 Peri-parturient rise (PPR) of faecal worm egg count

During late pregnancy and early lactation ewes experience a rise in faecal worm egg counts (PPR) (Beasley et al., 2010) which has been linked to the fact that ewes exhibit a range of impaired manifestations of resistance including the ability to resist establishment of newly acquired larvae (O'sullivan and Donald, 1973), the ability to suppress worm fecundity and in particular, the ability to expel adult worms (O'sullivan and Donald, 1970, 1973). Therefore, during this time periparturient ewes are considered as the largest contributor to pasture contamination with worm eggs which is a risk factor for the newborns. A number of studies have reported that the magnitude of the PPR can be regulated by the dietary supply of metabolisable protein (Donaldson et al., 2001) and the host genotype (Woolaston, 1992).

3.5 Role of Mg on immune function

A sufficient Mg intake is important for balanced immune response (Brandao et al., 2013), as Mg is involved in complement activation, phagocytic functions, controlling of apoptosis and programmed cell death, adjusting the production of free radicals and proinflammatory cytokines and signal transduction pathways of B and T lymphocytes (Brandao et al., 2013; Son et al., 2007). Furthermore, the role of Mg in lymphocyte signalling pathway activation has been previously reported in human patients diagnosed with a mutation in a Mg transporter gene MagT1 (novel X-linked human immunodeficiency). This condition is characterized by hypomagnesaemia, CD4+ lymphopenia and defective T-lymphocyte activation (Li et al., 2011). Along the same lines, in a mouse model with a specific T-cell deletion of another Mg transporter TRPM7, the T lymphocyte development was blocked

at the CD4+ and CD8+ stage, resulting in decreased CD4+ and CD8+ cells in the thymus (Jin et al., 2008). Moreover, knockout of TRPM7 in chicken lymphocyte cell line stopped the lymphocyte proliferation activity in vitro, however, when Mg was added to the culture medium the cells resumed their proliferation (Sahni et al., 2010). These observations suggest that Mg is essential for T lymphocyte development and proliferation.

Additionally, tumoricidal activities of splenic natural killer cell and peritoneal macrophage in mineral exposed rats were significantly increased after Mg and Mn supplementation (Son et al., 2007). Moreover, percentage of dead macrophages was reduced in the supplemented animals.

In farm animals, the influence of Mg supplementation on immune response has been reported recently in ewes (Ataollahi, 2018), the mentioned author concluded that maternal supplementation boosted the immune response in lambs as demonstrated by an increase in the total antioxidant capacity and oxidative burst response of leukocyte cells, furthermore studies in pigs have shown that Mg supplementation decreases piglet mortality and increases survivability at weaning time (Trawńska et al., 2013; Zang et al., 2014).

3.6 Hypotheses and aims of the PhD project

3.6.1 Hypotheses

Transition from late pregnancy to early lactation is characterized by high energy demand, nutrient deficiency, mineral imbalance and hormonal fluctuations which could interfere negatively in general with body homeostasis and particularly with the immune system, thus the main features of this period are the metabolic disorders and immune dysregulation. Since Mg is required for more than 600 metabolic reactions in addition to its role as an immune modulator, the following two hypotheses are proposed for this PhD study:

1. Mg supplementation during the transition period of ewes modulates mineral homeostasis, improves glucose metabolism and insulin function.
2. Mg supplementation alters distinct immunological parameters such as blood leukocyte composition, neutrophil phagocytosis, lymphocyte proliferative capacity, and the response to vaccination in periparturient ewes.

3.6.2 Aims

The main aims of this PhD project were:

1. To study the influence of Mg supplementation during the transition period on ewes' metabolic profile, such as serum levels of:
    - Ca, Mg and P_i
    - Glucose, beta hydroxybutyrate, total protein
    - Insulin and cortisol
    - Glucose (GLUT 1,3,4) and Mg transporter (SLC41A1, CNNM2, TRPM6, TRPM7, and MagT1) genes expression in ovine leukocytes
2. To investigate the effect of Mg supplementation during the transition period on ewes' selected immune parameters and mechanisms:
    - Innate immunity
        - Neutrophil phagocytosis *in vitro*
        - Composition of blood monocyte subpopulations
    - Adaptive immunity:
        - Lymphocyte proliferation *in vitro*
        - Response after vaccination against *Mycobacterium avium subsp. paratuberculosis*

4 Experimental setup – Animals & diets

This project was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) (Permission no.: 33.19-42502-05-18A359). All procedures involving animals were conducted in compliance with European Union guidelines concerning the protection of experimental animals.

4.1 Preliminary work

Well established immunological protocols in bovine were adapted to investigate ovine phagocytic activity *in vitro* and monocytes subset, furthermore those parameters as well as some serum biochemical parameters were compared between pregnant (n = 5) and nonpregnant ewes (n = 5), to get an idea regarding the influence of the transition period in the investigated parameters. Ten German black headed mutton (GMB) ewes were used in this preliminary work, for the main experiment the same breed was used. More information regarding the preliminary results is provided in the general discussion and in the appendices section.

4.2 Animals

4.2.1 Ewes as large animal model

According to the animal welfare-based reasons (3R principles) (Russell and Burch, 1959) the number of animals used in this PhD project was kept as low as possible. Using sheep as a model animal for ruminant system is suitable for several reasons, for instance they are less expensive to purchase and feed than cattle, moreover the two species have high similarity regarding the diet digestibility (Chishti et al., 2019; NRC, National Research Council, 2001). Furthermore, the metabolic changes and the hormonal fluctuations during the TP are highly comparable in both species. More importantly, selecting German black headed mutton breed to perform this experiment provides the most suitable model since this breed is known to be high susceptible to insulin resistance and pregnancy toxaemia during late pregnancy (Duehlmeier et al., 2011), therefore the selected animals are the most ideal model to investigate the role of Mg supplementation in insulin function and glucose metabolism during the TP.

4.2.2 Synchronisation protocol and pregnancy diagnosis

Twenty-three GBM ewes (entering the 2nd and 3rd lactation), were synchronised and mated naturally with three rams from the same breed. At day 39 post-mating, ewes were

transabdominally scanned for pregnancy. Out of the twenty-three ewes, nineteen became pregnant. The nineteen pregnant ewes were randomly divided into two groups: control group (n = 9) and Mg group (n = 10). The ewes were housed in the facilities of the Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Mecklenhorst, Germany. More information about the animals and the synchronisation protocol is provided in the first manuscript.

4.3 Diets

To cover the transition period, ewes started to receive the experimental diet one month ante-partum and continually till one month post-partum (Figure 3). The animals were offered two meals per day, in the morning 3 kg grass silage/animal (32% DM) and at noon 500 g pelleted concentrate/animal (89% DM). After parturition (48 h post-partum), the pellets were increased up to 1200 g/animal/day. The control group received approximately 2.97 g/animal/day (≈ 0.21% DM) of Mg during the antepartum (a.p.) period and 4.96 g/animal/day (≈0.24% DM) of Mg during postpartum period (p.p.) slightly above the recommendations of the National Research Council (NRC, National Research Council, 2007). The ration of the Mg group was additionally supplemented with Mg oxide (mixed with concentrate) resulting in a calculated daily Mg intake of 4.19 g/animal (≈ 0.30% DM) a.p. and 7.74 g/animal (≈ 0.38% DM) p.p., respectively. Ewes were allowed to adjust to this diet for two weeks before starting the experiment (adaptation period). The groups were housed separately with water available *ad libitum*. Detailed information on the feeding plan, components and composition of the applied diets is given in the first manuscript.

4.4 Blood sampling and vaccination

Blood was obtained by jugular vein puncture into K2E (EDTA), sodium heparin, and clot activator vacutainer tubes at five time points: d 30 a.p., d 14 a.p., d 1 p.p., d 14 p.p. and d 30 p.p. at 08:00 before the morning feeding (Fig.3).

At d 14 a.p., the ewes in both groups were injected subcutaneously with 1 mL of a commercial vaccine against *Mycobacterium avium paratuberculosis* (MAP). Whole blood samples were collected immediately before the injection (0) and 24 h following the vaccination (1), to assess the vaccination-associated changes in the composition of blood leukocytes. At d 0, d 7 and d 21 post vaccination serum samples were taken to determine the level of MAP-specific antibodies (MAP Abs) (Fig.3), more details are given in the second manuscript.

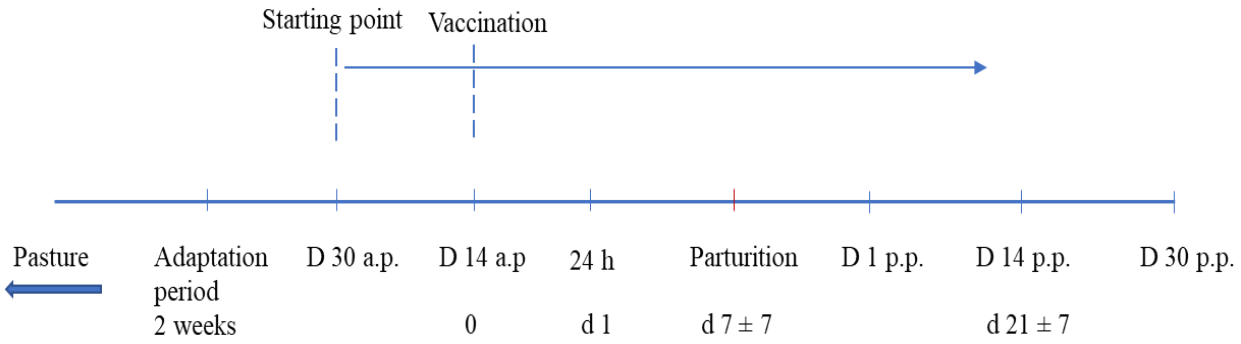

Figure 3. Blood sampling and vaccination time points during the transition period.

D & d: Day, a.p: ante-partum, p.p.: post-partum.

5 Manuscript 1

Serum parameters related to mineral homeostasis and energy metabolism in ewes kept on different dietary magnesium supply during the transition period

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Serum parameters related to mineral homeostasis and energy metabolism in ewes kept on different dietary magnesium supply during the transition period

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Abstract:

The present study investigated the effects of dietary magnesium supplementation on mineral status and intermediary metabolism of ewes during the transition period (TP). For this purpose, 19 pregnant ewes (2nd and 3rd lactation) were divided into a control group (Con, n = 9) kept on a daily magnesium intake of approximately 3.0 g ante-partum (a.p.) and 5.0 g post-partum (p.p.), and a magnesium group (Mg, n = 10, 4.2 g a.p., 7.7 g p.p.). Blood was collected at day (d) 30 a.p., d 14 a.p., d 1 p.p., d 14 p.p. and d 30 p.p., respectively. Serum levels of total magnesium were not affected by the treatment. In Con animals, serum levels of total calcium and phosphate were lower at d 1 p.p. in comparison to d 30 a.p. ($P \leq 0.05$). Only in Con ewes, serum glucose levels showed significant fluctuations throughout the entire observation period ($P < 0.05$) and were correlated negatively with those of beta-hydroxybutyrate (r^2: 0.70; $P < 0.01$, r^2: 0.76; $P < 0.01$) and positively with insulin (r^2: 0.46; $P < 0.05$, r^2: 0.59; $P < 0.05$) on d 30 and 14 a.p., respectively. At d 30 p.p., serum insulin levels were lower in the Mg group compared to the Con group ($P < 0.05$). As serum concentrations of calcium, phosphate and glucose seemed more stable throughout the TP, magnesium supplementation might be an approach to promote metabolic health in ewes.

Keywords: Dietary magnesium, minerals, glucose, insulin, transition period, ewes.

Highlights:

- Magnesium supplementation kept serum levels of calcium, phosphate and glucose stable throughout the transition period in ewes.
- Serum glucose levels correlated negatively with beta-hydroxybutyrate and positively with insulin levels during the ante-partum period only in the control group.
- Magnesium supplementation had no effect on serum protein fractions and cortisol levels.

1. Introduction:

The most crucial period for farm animals is the transition from late pregnancy to the onset of lactation (TP). In this period, the energy demand significantly increases to meet the requirements for the fetus and for production of colostrum and milk (Bell, 1995; Yildiz et al., 2005). Consequently, the mother's metabolic and endocrine profiles change dramatically (Ospina et al., 2010; Sordillo et al., 2009). Similar to other farm animals, pregnant ewes undergo metabolic and hormonal changes to meet the fetus's needs during late pregnancy until early lactation (Campion et al., 2016). Minerals such as calcium (Ca), magnesium (Mg) and phosphate (P_i) play an important role in the mineralisation of the skeletal system. Hence, it issuggested that ewes have a high demand for these minerals in late gestation when the fetal skeleton is mineralised. During lactation, milk production induces high drainage of minerals and energy from the blood into the mammary gland due to the high concentrations of glucose and minerals in sheep milk compared to other domesticated mammals (Balthazar et al., 2017). Accordingly, during the transition period ewes become more susceptible to a wide range of metabolic disorders such as pregnancy toxaemia (Duehlmeier et al., 2013a), hypocalcaemia and hypomagnesaemia (Brozos et al., 2011).

In humans, Mg deficiency can be associated with pancreatic beta-cell dysfunction, insulin resistance, type 2 diabetes mellitus, disorders of vitamin D metabolism and resistance to parathyroid hormone (PTH) (Barbagallo and Dominguez, 2007; Kostov, 2019). In veterinary medicine, the use of Mg as a dietary supplementation is a new trend to promote the animals' metabolic status, that has been investigated in ewes (Ataollahi, 2018), cows (Bach et al., 2018; Leno et al., 2017; Tebbe et al., 2018) and horses (Chameroy et al., 2011; Winter et al., 2018).

At the cellular level, Mg is involved in more than 600 metabolic reactions (De Baaij et al., 2015). Since kinases, ATPases, guanylyl cyclases and adenylyl cyclases depend on Mg-ATP for proper function, the role of Mg extends far beyond DNA and protein synthesis, DNA repair and glycolysis (De Baaij et al., 2015). Thus, Mg plays a vital role in virtually every cellular process.

Moreover, Mg is involved in the activation of insulin receptors (phosphorylation of tyrosine kinase) and participates in glucose transport across the cell membrane (Goldman and Fisher, 1983). Magnesium is required for PTH secretion and action. Additionally, it is also essential for renal 1α-hydroxylase activity, which is responsible for the production of the biologically active form of vitamin D, 1, 25- dihydroxyvitamin D (Rude et al., 2009).

Although the underlying mechanisms are not known, plasma concentrations of total Mg are kept within the range of 0.9-1.2 mmol/L, provided that the influx via absorption from the forestomachs (rumen and reticulum) into the extracellular space is larger than the efflux into the soft tissue and bones for fetal growth during pregnancy, milk production, and intestinal and urinary endogenous secretion (Martens et al., 2018). Mobilisation of Mg from bone is unlikely because the ratio between Ca: Mg is 42:1 which would disrupt Ca homeostasis (Fontenot et al., 1989). Therefore, absorption from the forestomach is probably the key factor determining plasma Mg levels, which can only be kept constant when the daily requirement is adequately balance by ruminal absorption (Martens et al., 2018).

It was shown that Mg requirements significantly increase during late pregnancy and early lactation with a greater risk of developing subclinical or clinical hypomagnesaemia in ewes (Brozos et al., 2011). Hypomagnesaemia induced by feeding a low Mg/high K diet reduced insulin responsiveness and glucose disposal (Matsunobu et al., 1990) and stimulated lipolysis in sheep (Sano et al., 1999). In contrast, ewes supplemented with Mg during the transition period showed a higher efficiency in energy regulation as observed by a decreased in plasma levels of non-esterified fatty acids (NEFA) concentration (Ataollahi, 2018). Similar findings were obtained in periparturient cows (Leno et al., 2017). In this context, German black headed mutton (GBM) ewes are recognized as being highly susceptible to pregnancy toxemia as diagnosed by elevated plasma levels of NEFA levels and insulin resistance during late pregnancy (Duehlmeier et al., 2011). Therefore, this knowledge led us to hypothesise that, Mg supplementation during the transition period modulates glucose metabolism and insulin function as well as mineral homeostasis in GBM ewes.

2. Materials and Methods

2.1. Ethical Statement

This study was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) (Permission no.: 33.19-42502-05-18A359). All procedures involving animals were conducted in compliance with European Union guidelines concerning the protection of experimental animals.

2.2. Synchronisation protocol and pregnancy diagnosis:

Twenty-three GBM ewes (entering the 2nd and 3rd lactation), were synchronised with Chronogest CR intravaginal sponges (20 mg, flugestone acetate, MSD Santé Animale, Beaucouzé, Cedex, France), which were inserted for 12 days. The sponges were removed at day 12 and at the same time the ewes were injected s.c. with 400 IE Pregmagon (Horse serum gonadotropin, IDT Biologica GmbH, Dessau-Rosslau, Germany). Within the next two days, the ewes were mated naturally with three rams from the same breed. At day 39 post-mating, ewes were transabdominal scanned for pregnancy by using a diagnostic scanner (Convex/Linear Ultrasonic system HS- 1600V, HONDA ELECTRONICS CO., LTD, Toyohashi, Japan). Out of the twenty-three ewes, nineteen became pregnant.

2.3. Animals and feeding regimes

The nineteen pregnant ewes were divided randomly into two groups: control group (Con, n = 9) and Mg group (Mg, n = 10). The ewes were housed in the facilities of the Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Mecklenhorst, Germany. Feed samples (silage and pellets) were analysed by the accredited service laboratory of the Lower Saxony Chamber of Agriculture, LUFA Nord-West, Oldenburg, Germany (Table 1). Analysis for minerals was performed by the Institute of Animal Nutrition, University of Veterinary Medicine Hannover, Foundation, by atomic absorption spectrometry (calcium, sodium, magnesium and potassium) and by spectrophotometry according to Schulz (2019) (phosphate).

The ewes were group-fed under surveillance of the personnel. Throughout the experimental period, there were no significant refusal. The animals received grass silage (3 kg per animal and day, 32% dry matter, DM) and concentrate (500 g per animal and day, 89% DM). While the Con group was fed a common concentrate available for sheep (Raiffeisen Schafe S2 lose, Agravis Niedersachsen-Süd GmbH, Hannover, Germany) containing 0.29% Mg (as fed), the pellets for the Mg group had been additionally supplemented with magnesium oxide to a final content of 0.51% (as fed). After parturition (48 h post-partum), the amount of concentrate was increased to 1200 g per animal and day (as fed). Ante-partum, the

estimated daily Mg intake was 2.97 g per animal in the control group (0.21% of DM) and 4.19 g per animal in the Mg group (0.30% of DM). Postpartum, the estimated daily Mg intake was 4.96 g per animal in the control group (0.24% of DM) and 7.74 g per animal in the Mg group (0.38% of DM).

Ewes were allowed to adjust to this diet for two weeks the experiment was started (adaptation period). The groups were housed separately with water available *ad libitum*.

Table 1
Composition of silage and pelleted concentrate diets in % per dry matter (DM) if not stated otherwise.

	Grass silage	**Concentrate**
Crude protein	15.0%	20.5%
Crude fat	-	4.66%
Crude fibre	25.8%	10.8%
Acetic acid	0.12%	-
Butyric acid	1.20%	-
Lactic acid	2.19%	-
Metabolisable energy (MJ/kg DM)	10.7	11.6
Minerals		
Calcium	0.58%	1.99%
Phosphorus	0.32%	0.84%
Sodium	0.11%	0.29%
Magnesium	0.16%	0.32%/0.58%*
Potassium	3.15%	1.12%

Pelleted concentrate components: Rapeseed meal (extracted) soapstock 19.7%, Palm kernel cake 15.0%, wheat bran 13.5%, Wheat semolina bran 12.3%, Molasses sugar beet

pulp 7.5%, Dried *distiller grains* with solubles (DDSG) 7.2%, Calcium carbonate 3.8%, Whey concentrate (partly desugared) 3.5%, Rye bran 3.0%.
*Pellets for the control group contained 0.32%, pellets for the Mg group 0.58% magnesium.

2.4. Blood sampling

Blood was obtained by jugular vein puncture into K2E (EDTA) and CAT (Clot Activator Tube) vacutainer tubes (BD Vacutainer, Belliver Industrial Estate, Plymouth, UK) at five time points: d 30 a.p., d 14 a.p., d 1 p.p., d 14 p.p. and d 30 p.p. at 08:00 before the morning feeding. Blood samples were transferred to the diagnostic laboratory of the Clinic for Swine, Small Ruminants and Forensic Medicine University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. Serum was separated by centrifugation and stored at -20 °C for further analysis.

2.5. Biochemical determinations

Serum levels of total Ca were measured using a commercial kit (Labor + Technik LT-SYS, Labor + Technik Eberhard Lehmann GmbH, Berlin, Germany) spectrophotometrically (Biotek Instruments Inc. Uvikon XL UV-Visible, Scanning spectrophotometer, Winooski, VT, USA). Total Mg, phosphate, glucose, total protein, and albumin concentrations in the serum samples were determined by using commercial kits (Labor + Technik LT-SYS, Labor + Technik Eberhard Lehmann GmbH, Berlin, Germany). Beta-hydroxybutyrate levels in the serum were determined with a commercial kit (Randox Ranbut, Randox Laboratories Ltd. London, United Kingdom) and quantified using a biochemical analyser (Cobas Mira Plus, Automated, Roche Diagnostics International AG, Basel, Switzerland). Serum levels of globulin were calculated by subtracting the albumin values from the total protein values. Serum levels of insulin were determined with an immunoradiometric assay (IRMA) kit (Beckman Coulter, Prague, the Czech Republic) in the Endocrinology Laboratory, Clinic for Cattle, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. The insulin sensitivity index was determined as quantitative insulin sensitivity check index (QUICKI) according to Katz et al, (2000).
$QUICKI = 1/ [\log (\text{glucose (mg } dL^{-1})) + \log (\text{insulin } (\mu U\ mL^{-1}))]$
Serum levels of cortisol were estimated using ABNOVA® Sheep cortisol ELISA kits (Abnova, Taoyuan, Taiwan), which were applied in accordance with the manufacturer's instructions.

2.6. Statistical analysis

Data are expressed as means ± SEM; n represents the number of animals per group. The unpaired t-test, ANOVA for repeated measures and Sidak's multiple comparisons test (GraphPad Prism 8 Software, San Diego, CA, USA) were used for comparison between the different time points and groups. Correlations between selected parameters (glucose-BHB and glucose-insulin) were analyzed by linear regression analysis. Differences were considered statistically significant when $P < 0.05$, and $P < 0.1$ was used to define trends.

3. Results

The effects of the transition period and dietary Mg supplementation on the serum mineral concentrations, intermediary metabolism parameters, proteins, insulin and cortisol levels are shown in Figures 1-5. Forty lambs were born, (control group = 19 lambs, Mg group = 21 lambs). Birth type per group (single, twins and triplets) per group are shown in Table 2.

Table 2
Birth type per group.

Group	Single	Twins	Triplets	Total
Control	2	4	3	19
Mg	1	7	2	21

3.1. Minerals

In both groups, neither the time nor the treatment had a significant effect on the serum levels of total Mg (Fig.1-Magnesium). Control ewes showed a significant decrease ($P < 0.05$) in the serum levels of total Ca at d 1 p.p. (2.4 ± 0.07 mmol/L) compared to d 30 a.p. (2.7 ± 0.10 mmol/L), while the respective values were stable throughout TP in Mg ewes (Fig. 1-Calcium). In both groups, serum level of total P_i was lower during the ante-partum period compared to the post-partum period, Interestingly, serum level of P_i was significantly increased on d 14 p.p. in comparison to d 30 a.p. in the Mg group ($P < 0.05$)

(Fig. 1- Phosphate). A sharp decrease was observed at d 1 p.p. (1.12 ± 0.05 mmol/L) (P < 0.05) only in the control ewes (Fig. 5- A-Phosphate, day 1 p.p.).

3.2. Serum intermediary metabolism parameters

In the control group, serum levels of glucose were significantly lower (P < 0.05) at d 30 a.p and d 14 a.p. (3.23 ± 0.17 and 3.1 ± 1.16 mmol/L, respectively) before parturition, then increased gradually and peaked at d 30 p.p. (4.1 ± 0.13 mmol/L). In the Mg group, the lowest values were also observed before lambing (d 30 a.p.: 3.34 ± 0.27 mmol/L & -14 days: 3.31 ± 0.07 mmol/L) and on d 1 p.p. (3.23 ± 0.16 mmol/L). Afterwards, the highest values were reached already at d 14 p.p. (3.61 ± 0.10 mmol/L), resulting in trend for interaction of time and group (P = 0.064) (Fig. 2- Glucose). On d 1 p.p. glucose concentrations in the Mg group showed a trend (P = 0.07) to be decreased compared to control ewes (Fig. 5-A-Glucose, day 1 p.p.); this difference became significant on d 30 p.p. (P < 0.05) (Fig. 5- B-Glucose, day 30 p.p.).

Serum levels of beta-hydroxybutrate showed the highest value at d 14 a.p in the control group (0.68 ± 0.17 mmol/L) and at d 14 p.p. in the Mg group (0.78 ± 0.07 mmol/L) (P > 0.05), (Fig. 2 - Beta-hydroxybutrate). A negative correlation was observed between glucose and beta-hydroxybutrate levels during a.p. only in the control group (r = - 0.74, P < 0.001) (Table 3).

In both groups, the lowest serum levels of insulin were found before lambing (d 30 and d 14 a.p.); the control group values were 24.43 ± 3.6 µU/mL and 21.9 ± 4.4 µU/mL, respectively. The Mg group values were 22.47 ± 3.4 µU/mL and 19.42 ± 2.1 µU/mL, respectively. Insulin values increased gradually and peaked on d 30 p.p. (52.85 ± 5.6 µU/mL) in the control group and on d 14 p.p. (40.57 ± 6.8 µU/mL) in the Mg group (P < 0.05) (Fig. 2- Insulin). Interestingly, on d 30 p.p., the insulin values were lower in the Mg ewes (32.90 ± 4.3 µU/mL) compared to the control ewes (52.85 ± 5.6 µU/mL) (P < 0.01) (Fig. 5 -B- Insulin, day 30 p.p.). During the ante-partum period a positive correlation was observed between glucose and insulin levels only in the control group (Table 3).

In the control and Mg ewes, the highest values of QUICKI were obtained during the post-partum period d 30 p.p. (2.23 ± 0.04) and d 14 p.p. (2.11 ± 0.07), respectively (Fig. 2- QUICKI). At d 30 p.p., a significant difference (P < 0.05) was observed between the control and Mg group, ewes offered more Mg having lower value compared to the control ewes (Fig. 5- B-QUICKI, day 30 p.p.).

3.3. Protein parameters

In both groups, serum levels of total protein and albumin showed the same pattern throughout TP (Fig. 3- Total protein, Albumin, and Globulin). The lowest values of total protein and albumin were reported at d 1 p.p. In the control group, the values were 58.9± 1.3 g/L and 30.23± 0.78 g/L, respectively ($P < 0.05$- 0.0001), in the Mg group, the values were 61.1±1.4 g/L and 28.43± 1.3 g/L, respectively ($P < 0.05$ - 0.0001) (Fig. 3-Total protein and Albumin). For the serum levels of globulin, the control ewes showed lower values throughout the TP compared to the Mg ewes ($P > 0.05$). In both groups, the lowest globulin values were reported at d 14 a.p. (26.5 ± 0.96 g/L and 30.4 ± 1.9 g/L, respectively). Serum levels of globulin increased gradually to reach the highest value at d 30 p.p. (30.96 ± 0.84 g/L) in the control group and at d 14 p.p. (33.9 ± 2.3 g/L) in the Mg group ($P< 0.05$ - 0.001) (Fig.3-Globulin).

3.4. Cortisol levels

In both groups, neither the time nor the Mg supplementation had significant effects on the serum levels of cortisol throughout the TP. The obtained values showed huge variations between the animals. In both groups, the lowest values were reported at d 14 a.p. (control group: 72.3 ± 6.4 ng/mL; Mg group: 104.8 ± 29.7 ng/mL), while the highest values were recorded at d 30 p.p. in the control group (221.9 ± 121.95 ng/mL), and at d 14 p.p. in the Mg group (239.9 ± 66.4 ng/mL) (Fig. 4-Cortisol).

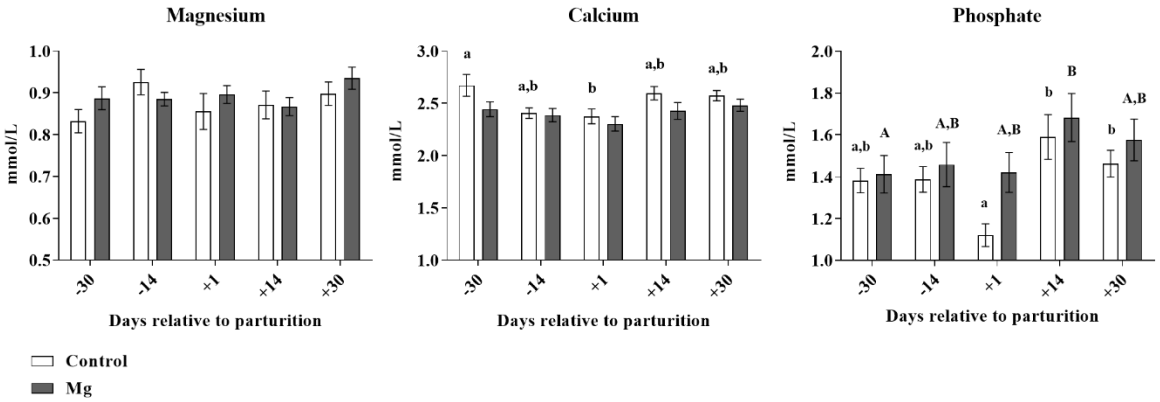

Fig. 1. Serum mineral levels (Ca, Mg and P_i). Repeated measurements two-way ANOVA (time and group) revealed no group effect, however, effects of time on Ca and Ca/Mg ($P < 0.01$) and Pi ($P < 0.001$) were observed. Significant time-dependent differences ($P < 0.05$) in the Sidak post-test are indicated by different small letters for the control group and capital letters for the Mg group. Control group n = 9, Mg group n = 10, mean ± SEM.

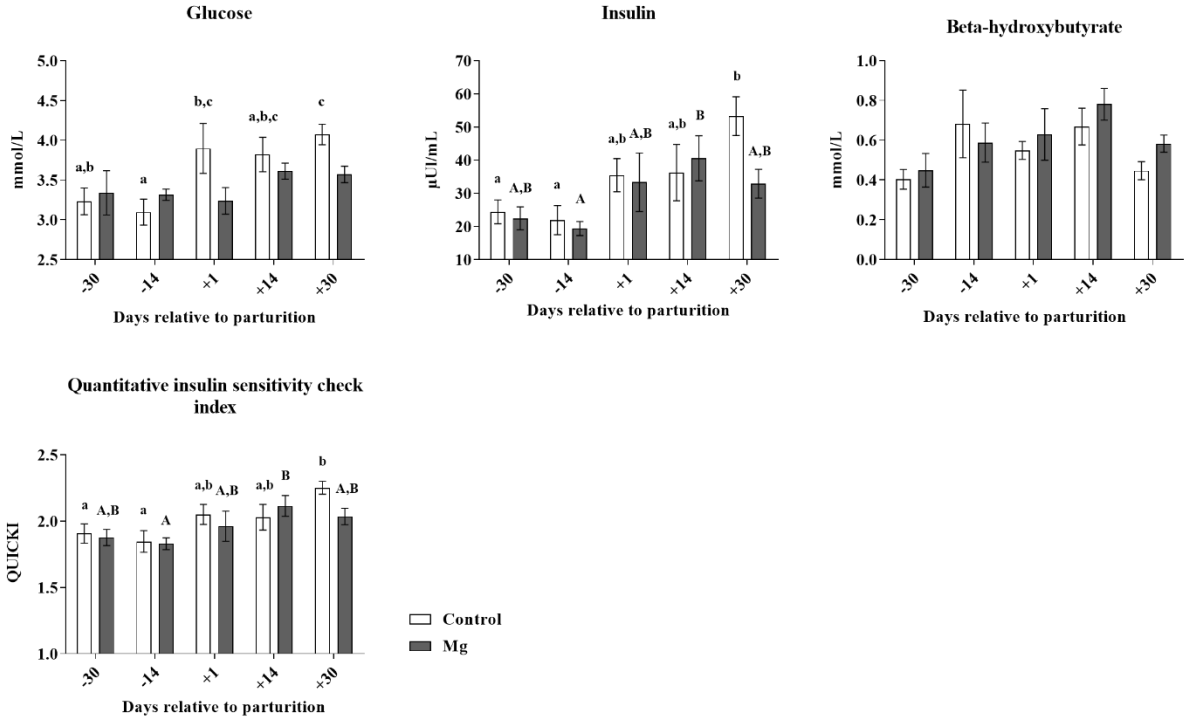

Fig. 2. Serum glucose, beta-hydroxybutyrate, insulin levels and QUICKI. Repeated measures ANOVA (time and group) revealed no group effect, however, effects of time on glucose and beta-hydroxybutyrate ($P < 0.01$) were observed for glucose, moreover a tendency of interaction ($P = 0.064$) was reported in glucose. Furthermore, insulin and QUICKI were influenced by time as well ($P < 0.001$). Significant time-dependent differences in the Sidak post-test are indicated by different small letters for the control group and capital letters for the Mg group. Control group n = 9, Mg group n = 10, mean ± SEM.

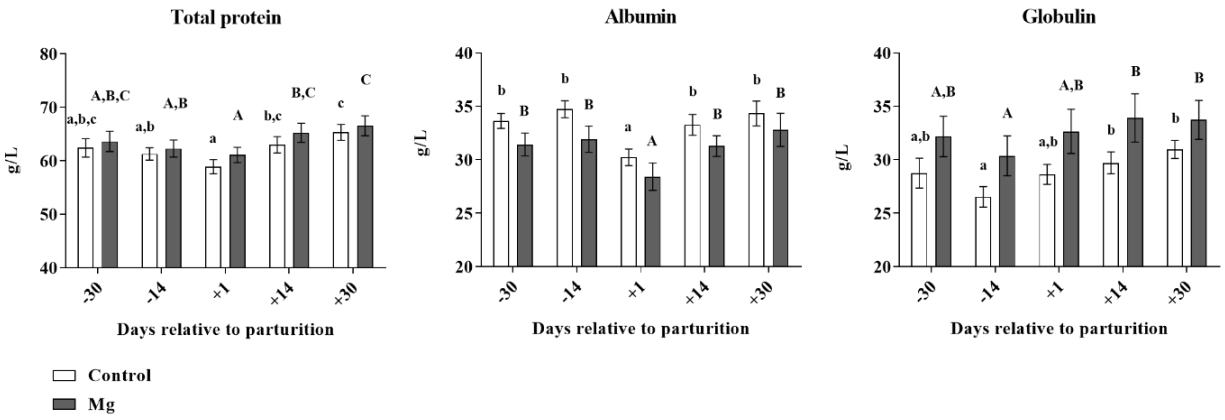

Fig. 3. Serum total protein, albumin and globulin levels. Repeated measures ANOVA (time and group) revealed no group effect, however, effects of time in total protein, albumin and globulin ($P < 0.0001$) were observed in both groups. Significant time-dependent differences in the Sidak post-test are indicated by small letters for the control group and capital letters for the Mg group. Control group n = 9, Mg group n = 10, mean ± SEM.

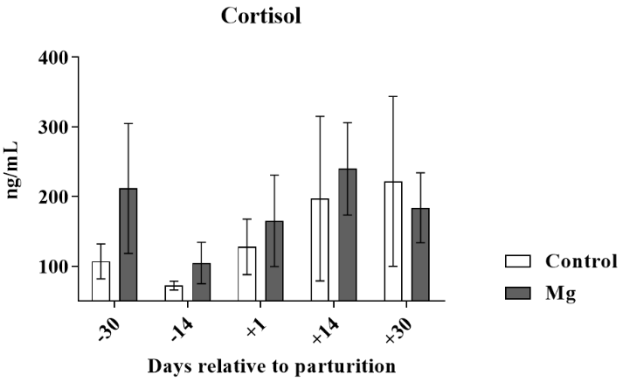

Fig. 4. Serum cortisol levels. Repeated measures ANOVA (time and group) revealed neither the time nor the group had a significant effect on cortisol levels during TP. Control group n = 5, Mg group n = 5, mean ± SEM.

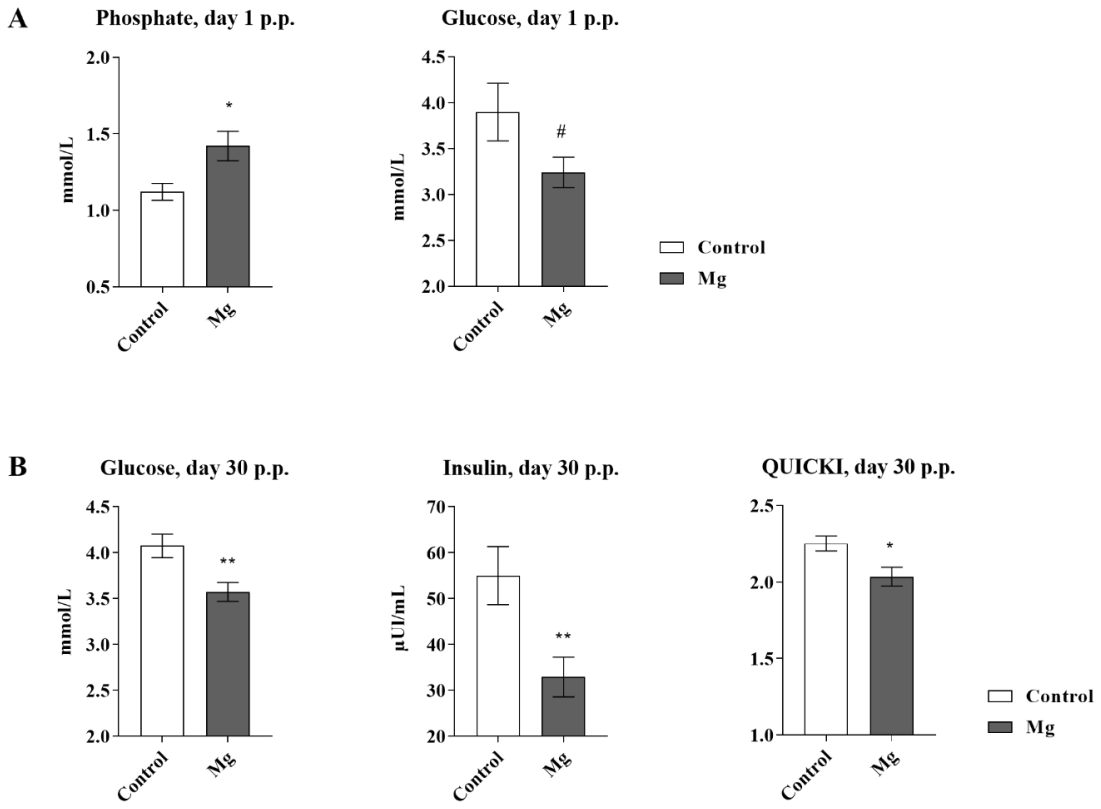

Fig. 5. Serum phosphate, glucose, insulin and QUICKI (Quantitative insulin sensitivity check Index) on day 1 p.p. (A) and day 30 p.p. (B). Unpaired t-test, Control group n = 9, Mg group n = 10, mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P = 0.07$.

Table 3

Correlations between glucose and beta-hydroxybutyrate (BHB) as well as glucose and insulin on day 30 and 14 ante-partum (a.p.) revealed by linear regression analysis.

	Control group	**Mg group**
BHB		
Day 30 a.p.	$y = (-0.25 \pm 0.06)\ x + 1.20 \pm 0.20$ $P < 0.01$; r^2: 0.70	n.s.
Day 14 a.p.	$y = (-0.90 \pm 0.19)\ x + 3.48 \pm 0.60$ $P < 0.01$; r^2: 0.76	n.s.
Insulin		
Day 30 a.p.	$y = (14.5 \pm 5.85)\ x + 22.4 \pm 19.4$ $P < 0.05$; r^2: 0.46	n.s.
Day 14 a.p.	$y = (20.7 \pm 6.46)\ x + 42.2 \pm 20.2$ $P < 0.05$; r^2: 0.59	n.s.

5. Discussion

The aim of this study was to investigate the effect of dietary Mg supplementation on the mineral status and intermediary metabolism parameters during the TP in ewes. Since the animals were kept in a common free stall, it was impossible to calculate the individual dry matter intake (DMI). In this study, it was demonstrated that the TP had significant effects on serum mineral profile and intermediary metabolism parameters in ewes as an adaptive physiological mechanism associated with rapid fetal growth and lactogenesis. These findings are comparable to previous studies in different farm animals (Antunović et al., 2011; Bell, 1995; Wilkens et al., 2014; Yildiz et al., 2005). Furthermore, it was shown that dietary Mg supplementation has a modulatory effect on serum levels of Ca, P_i, glucose and insulin during the TP.

The classical implication of high K intake for the inhibition of Mg absorption is well known in ruminants (Fontenot et al., 1960; Schonewille et al., 1999). In the present study, the daily K intake (35.6 g a.p. and 42.6 g p.p.) from silage (3.15% of DM) and pellets (1.12% of DM) was higher than recommended by the NRC (2007). However, there were no indications for insufficient Mg supply or absorption in the Con group. As long as Mg

absorption exceeds the requirements, physiological serum concentrations are maintained (Ram et al., 1998). In fact, the daily intake of Mg exceeded the recommendations for 80 to 100 kg ewes with twins or triplets in late gestation (2.3 to 3.0 g) and in early lactation (3.0 to 4.1 g) made by the NRC (2007). Although the intake in the control ewes was more than adequate, too (approximately 3.0 g a.p. and 5.0 g p.p.), we expected to find beneficial effects of a surplus of Mg in line with previous studies done in periparturient cows and ewes (Ataollahi, 2018; Bach et al., 2018; Leno et al., 2017; Tebbe et al., 2018).

In both groups, serum concentrations of total Mg, Ca and P_i were within the reference range described for this breed (Bickhardt and König, 1985) and within the normal range reported for sheep (Radostits et al., 2006). Serum levels of total Mg remained stable throughout the TP in both groups. Similar findings were reported for cows (Kulcu and Yur, 2003). In contrast, Yildiz et al. (2005) observed a significant decrease in serum levels of total Mg during the prepartum period in ewes. Although daily Mg intake was approximately 40% to 55% higher in the Mg group, serum Mg concentrations did not differ from those in the Con group. This could be related to Mg digestibility, absorption or potential interactions between the Ca and Mg transport mechanisms in the rumen. Furthermore, as any change in Mg balance is counterbalanced by corresponding changes in urinary excretion (Ram et al., 1998), excess Mg absorbed from the diet might simply be excreted via urine.

On the first day p.p., serum levels of total P_i decreased sharply ($P < 0.01$) in the control group. This finding is in line with a previous study in periparturient sheep, cows, and goats (Wilkens et al., 2014). However, some studies reported that the lowest P_i serum values were observed at late pregnancy in pregnant sheep (Sansom et al., 1982; Yildiz et al., 2005). Mg group showed stable serum levels of P_i on d 1 p.p. which was similar to a previous study in cows supplemented with Mg during TP (Leno et al., 2017). There are two explanations that could be used to interpret these results in the control ewes. Firstly, it could be due to high serum levels of cortisol (parturition stress) which is correlated negatively with the serum levels of Ca and P_i (Horst and Jorgensen, 1982). Moreover, cortisol could have an indirect effect leading to hyperglycemia and hyperinsulinemia which cause a shift in P_i from extracellular to intracellular space, resulting in hypophosphatemia (Grünberg et al., 2006; Taylor et al., 1991). From a previous study, Mg supplementation was reported to decrease cortisol levels (Dmitrašinović et al., 2016), and improve glucose metabolism and insulin function in humans (Mooren et al., 2011; Paolisso et al., 1994; Rodríguez-Morán and Guerrero-Romero, 2003). Secondly, Mg is involved in PTH and 1, $25(OH)_2$ D_3 function, which are essential for Ca and P_i homeostasis (Ataollahi, 2018; Rude et al., 2009).

It is well known that sheep develop hypocalcaemia more often during late gestation (Oetzel, 1988), while in cows and goats, hypocalcaemia was reported at parturition (Reinhardt et al., 2011; Wilkens et al., 2014). However, Yildiz et al. (2005) reported a significant decrease in serum levels of Ca close to parturition in ewes. In the present study, a slight decrease in serum levels of Ca ($P < 0.05$) was observed on d 1 p.p. in the control group.

In the current study, serum levels of glucose and beta-hydroxybutyrate were within the normal range in sheep in both groups according to respective reference values given for this breed (Bickhardt and König, 1999) and and the normal range of sheep (Radostits et al., 2006). While the insulin values were higher than what reported previously in periparturient ewes (Duehlmeier et al., 2011; Henze et al., 1998), which could be due to different methodical approaches and different dates of sampling. The low serum levels of glucose and insulin observed in both groups before parturition (d 30 and d 14 a.p.) could be associated with increased requirements for intensive fetal growth during late pregnancy. These findings are in line with previous studies (Duehlmeier et al., 2011; Regnault et al., 2004; Schlumbohm et al., 1997). The highest glucose serum value was reported at d 1 p.p. in the control group, could attributable to parturition stress which mediated by glucocorticoids and catecholamines (Chen et al., 1998), and it has been closely linked to liver gluconeogenesis activation and hyperglycaemia around parturition in cows (Vannucchi et al., 2015). Another explanation for the highest glucose value being present at d 1 p.p. could be due to the reduction in insulin sensitivity (Duehlmeier et al., 2013a; Duehlmeier et al., 2013b; Regnault et al., 2004; Van der Walt et al., 1980), which interferes with glucose tissue uptake. In the Mg group, serum levels of glucose were stable throughout TP in comparison to the control group. This observation could confirm the role of Mg in the glucose metabolism as reported previously in humans and hypomagnesemic sheep (Matsunobu et al., 1990; Meludu and Adeniyi, 2001). In ruminants, the major part of glucose must be synthesized by gluconeogenesis in the liver using propionate as main substrate (Aschenbach et al., 2010); where Mg is required as a coenzyme for most of the enzymes involved the gluconeogenesis (Voma and Romani, 2014). In addition, Mg participates in the transport of glucose in insulin-dependent mechanisms across the cell membrane (Rodríguez-Morán and Guerrero-Romero, 2003), which increases the tissue's uptake and insulin sensitivity, sequentially decreasing the insulin and glucose levels in the circulation. However, slight decrease was observed in QUICKI at d 30 p.p. in Mg ewes compared to control ewes, QUICKI is consider as a reproducible method to evaluate the

insulin sensitivity in humans (Katz et al., 2000). Normally, in ruminants the insulin sensitivity index is based on the serum concentrations of insulin, glucose, and the non-esterified fatty acids (NEFA) (Duehlmeier et al., 2013b), in the present study the NEFA values were missing. Therefore, the results have to be discussed carefully.

The highest serum levels of beta hydroxybutyrate were observed at d 14 a.p. and d 14 p.p. in control and Mg ewes, respectively. Similar to previous study done in GBM ewes (Duehlmeier et al., 2011) which reflects a metabolic challenge during this period as it close to the lambing time, however, no significant differences were observed between the two groups. Regarding the negative correlation between glucose and BHB as well as the positive correlation between serum levels of glucose and insulin in the control ewes before lambing, our findings are in accordance with what was reported previously by Duehlmeier et al, (2011). The lack of such correlations in the Mg group might indicate a modulation of glucose metabolism. Therefore, it could be speculated that ewes offered more Mg were able to some extent to maintain stable glucose levels throughout the TP compared to the control group.

Serum metabolites are known to be changed during pregnancy as a result of dam and fetal requirements (Batavani et al., 2006). In the current study, in both groups serum levels of total protein, albumin and globulin were within the normal range in sheep according to respective reference values given for this breed (Bickhardt and König, 1999) and the normal range of sheep (Radostits et al., 2006), the values showed the same pattern throughout the TP. At d 1 p.p., serum levels of total protein and albumin significantly decreased in both groups. The parturition process is considered as an inflammation response, accordingly, the dam´s liver is activated to produce inflammatory molecules such as positive acute-phase proteins (e.g. C-reactive protein) and impaired at the same time the production of the negative acute-phase proteins such as albumin (Trevisi et al., 2016). Therefore, we noticed a reduction in serum concentrations of total protein and albumin around parturition in both groups, which could be due to the competition for the substrates. Mg supplementation had no effects on protein parameters. However, ewes from the Mg group showed higher serum levels of globulin throughout TP compared to the control group ($P > 0.05$). The relation between Mg and protein homeostasis is not yet fully understood.

Around parturition, serum concentrations of cortisol in ewes significantly increase due to hypothalamo-pituitary-adrenal axis stimulation (Vannucchi et al., 2015). The serum levels of cortisol observed in the present study throughout the TP (except at d 14 a.p.) were substantially higher than what was reported for sheep during normal farm handling

procedures such as isolation from the flock and their lambs, restraining and blood sampling (73 ng/mL) (Hargreaves and Hutson, 1990). Even lower values (26.5±7.8 ng/mL) were reported previously in ewes at d 14 a.p. (Duehlmeier et al., 2013b). This substantial difference to our findings could be due to the fact that these animals had previously been provided with an indwelling catheter and that the blood samples were taken much more smoothly compared to our study. A prolonged restraint and isolation for 2 h were reported to increase the serum levels of cortisol up to 100 ng/mL (Apple et al., 1993). In the present study, huge variations in serum levels of cortisol were observed among the animals of both groups, which could be due to genetic factors and the individual response to stress during the blood sampling.

6. Conclusion

In the present study, changes in serum levels of Ca, P_i and glucose during the transition period were observed in the control ewes but not in the Mg ewes, suggesting an opportunity for strategic use of additional Mg in the transition period to promote metabolic health in GBM ewes. Further research addressing the relationship between different concentrations of Mg intake and insulin function in periparturient ewes is required.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Manuscript 2

Blood leukocyte composition and function in periparturient ewes kept on different dietary magnesium supply

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Collection of the blood samples: Mona H. Ahmed, Mirja R. Wilkens, Bernd Möller
Laboratory work: Mona H. Ahmed adapted well established protocols in bovine to investigate ovine neutrophil phagocytosis and lymphocyte proliferation *in vitro*, as well as mononuclear cells separation and monocyte subsets determination, additionally performed the analyses using flow cytometer software.
Statistic analysis: Mona H. Ahmed
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Blood leukocyte composition and function in periparturient ewes kept on different dietary magnesium supply

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Abstract

Background: Transition period (TP) is characterised by physiological and metabolic changes contributing to immunodysregulation. Since knowledge about this period in sheep is scarce, we analysed changes in selected immune parameters during the TP in ewes and whether dietary magnesium (Mg) supplementation could modulate these immune parameters. Pregnant ewes (2nd and 3rd lactation) were divided into a control group (CONT, n = 9) and a Mg group (MAG, n = 10) supplemented with Mg oxide resulting in a daily Mg intake of approximately 0.24% and 0.31% (MAG) of dry matter during ante- (a.p.) and post-partum (p.p.) periods, respectively. Blood samples were collected between days (d) 30 a.p. and d 30 p.p.. Whole blood neutrophil phagocytic activity, monocyte subset (classical cM, intermediate intM, non-classical ncM) composition and the proliferative capacity of lymphocytes were determined flow cytometrically. At d 14 a.p., all ewes were vaccinated against *Mycobacterium avium subsp. paratuberculosis* (MAP).

Results: Both groups showed a sharp increase in the total leukocyte counts (TLC) and neutrophil counts ($P < 0.0001$), at d 1 p.p., while, monocytes and their subpopulations

displayed the highest values at d 30 p.p. ($P \leq 0.05$). At d 1 p.p. the neutrophil phagocytic activity was higher ($P < 0.05$) in MAG ewes. Throughout the TP, the proliferative response of CD4+ cells was significantly higher in the MAG group ($P < 0.05$). Ewes in both groups responded with an increase in the TLC, neutrophil numbers ($P \leq 0.05$) and ncM ($P < 0.001$) 24 h post vaccination, whereas monocytes and cM dropped in numbers ($P \leq 0.05$). Numbers of intM only increased in MAG ewes ($P < 0.05$), whereas lymphocyte numbers decreased ($P < 0.01$). Mg supplementation did not affect the significant increase in MAP-specific antibodies at d 7 and 21 post vaccination. Total Mg and Ca serum levels did not show any differences between the two groups.

Conclusion: Whereas TP-associated fluctuations in blood leukocyte numbers are not influenced by Mg supplementation, neutrophil phagocytic activity, the proliferative capacity of CD4+ cells and the cellular response within 24 h after a vaccination are subject to modulation.

Keywords: magnesium, neutrophil phagocytosis, monocyte subsets, lymphocyte proliferation, vaccination, transition period, sheep.

Background

Dramatic changes are observed in the metabolic and endocrine profiles as well as the immune responses in farm animals during the transition period (TP) [1, 2] . Similar to other farm animals, pregnant ewes undergo metabolic, hormonal and immunological changes to accommodate the fetus's needs mainly during late pregnancy until early lactation [3]. Immune dysregulation occurs commonly during the TP, this being due to hormonal fluctuations (e.g. progesterone, cortisol, oxidative stress, negative energy balance and mineral imbalances [4, 5]. Changes in immune mechanisms during the TP were reported mainly in cows and to some extent in ewes, such as a reduction in phagocytic activity of neutrophils and macrophages, alteration in the composition of circulating monocyte subsets, production of cytokines, complement activation, proliferation of lymphocytes, and the production of antibodies [6-8]. Accordingly, cows show greater susceptibility to a wide range of diseases such as mastitis and retained placenta, while ewes become more vulnerable to gastrointestinal nematodes peri-parturient rise (PPR) in faecal egg counts [9]. During late pregnancy and early lactation ewes experience a rise in faecal worm egg counts [9-11] which has been linked to the fact

that ewes exhibit a range of impaired manifestations of resistance including the ability to resist establishment of newly acquired larvae [12], the ability to suppress worm fecundity [10, 12, 13] and in particular, the ability to expel adult worms [10]. A number of studies have reported that the magnitude of the PPR can be regulated by the dietary supply of metabolisable protein [14, 15] and the host genotype [16].

At cellular level, magnesium (Mg) is required for more than 600 metabolic reactions as a coenzyme or substrate [17]. Mg is an essential component of DNA replication and repair, RNA transcription, amino acid synthesis, and protein formation. In addition, Mg is an important regulator of many enzymes involved in glycolysis, such as adenine nucleotides [17]. It is well known that, Mg availability is of major importance for glucose metabolism and insulin function [18], as it has been observed in diabetic humans and hypomagnesaemic sheep [19-21]. Activation of cellular proliferation is initiated by growth factors that increase glucose uptake and protein synthesis [22], since Mg is a key factor in both processes and in the activation of mammalian target of rapamycin (mTOR) complex [23]. Thus, Mg is undoubtedly involved, in cell signalling and proliferation, which confirms its role as an immunomodulatory as reported recently [24-26].

Although the underlying mechanisms remain unknown, plasma Mg is kept within the range of 0.9-1.2 mmol/L, provided that the influx via absorption from the forestomachs region into the extracellular space is greater than the efflux into the soft tissue and bones for foetal growth during pregnancy, milk production, and intestinal and urinary endogenous secretion [27]. Mobilisation of Mg from bone is unlikely because the ratio Ca:Mg is 42:1 which would disrupt Ca homeostasis [28]. Therefore, absorption from the forestomach region is probably the key factor determining plasma Mg levels, which can only be kept constant when the daily requirement is adequately balanced by ruminal absorption [27]. In ewes, Mg requirement is increased significantly during late pregnancy and early lactation. Therefore, subclinical hypomagnesaemia could occur during the TP in ewes [29].

This knowledge led us to hypothesise that Mg supplementation during the TP of ewes modulates mineral homeostasis, improves glucose metabolism and insulin function as well as distinct immunological parameters such as blood leukocyte composition, neutrophil phagocytosis, lymphocyte proliferative capacity, and the response to vaccination.

Results

Blood leukocytes

At d 1 p.p., a significant increase was observed in TLC and neutrophil counts ($P < 0.0001$) (Fig. 1-A, B). This was similar in the control and Mg group. Lymphocyte numbers were significantly lower at d 14 a.p. in the Mg group ($P < 0.05$) (Fig. 1-C). Total numbers of monocytes and monocyte subpopulations (cM, intM and ncM) displayed the highest values at d 30 p.p. ($P \leq 0.05$) (Fig. 1-D, E, F, G).

Neutrophil phagocytic activity

The fraction of phagocytosis-positive neutrophils was lower during the ante-partum period (d 30 a.p. and d 14 a.p.) compared to the post-partum period (d 1 p.p., d 14 p.p. and d 30 p.p.) (Fig. 2-A) in both groups. At d 1 p.p. a higher percentage of phagocytosis-positive neutrophils was observed in the Mg group ($P < 0.05$) (Fig. 2-B). The mean phagocytic capacity per cell changed more clearly through the TP (Fig. 2-C) with lowest values in the ante-partum period in both groups ($P < 0.05$-0.0001). At d 1 p.p. ewes supplemented with Mg displayed significantly higher neutrophil phagocytic capacity ($P < 0.05$) compared to the control ewes (Fig. 2-D).

Lymphocyte proliferation

Neither the time nor the Mg supplementation had significant effects on the proliferative capacity of all lymphocytes throughout the TP (Fig. 3-A). However, Mg supplemented ewes showed a higher proliferative capacity of CD4+ T lymphocytes ($P < 0.05$) compared to the control ewes (Fig. 3-B). In addition, only in the Mg group there was a significant time dependent effect on the proliferative capacity of CD8+ T-cells ($P < 0.01$, Fig. 3-C).

MAP vaccination response

Transient enrichment in TLC and neutrophil numbers was observed in the peripheral blood within 24 h following the vaccination in both groups ($P < 0.001$ and $P < 0.0001$, respectively) (Fig. 4-A, B). However, a depletion was observed in monocyte ($P < 0.001$) and cM ($P < 0.0001$) numbers (Fig. 4-C, D). While ncM showed a significant increase ($P < 0.0001$) in response to vaccination in both groups (Fig. 4-F), intM numbers increased significantly ($P < 0.05$) only in the Mg group (Fig. 4-E). Interestingly, a significant decrease in lymphocyte numbers was observed within 24 h only in the Mg group ($P < 0.01$) (Fig. 4-G). In contrast, CD4+ and CD8+ T cells remained unaltered (Fig. 4- H, I).

The single vaccination dose successfully stimulated the existent memory cells in the peripheral blood as observed by a significant increase in the antigen-specific antibody (MAP Ab) activities at days 7 and 21 post vaccination in both groups ($P < 0.0001$) (Fig. 4-J). However, there were no significant differences between the two groups.

Faecal worm egg counts and eosinophil numbers

The faecal worm egg counts were obtained as total numbers/g faeces, the dectected nematode species were: Haemonchus, Trichostrongylus, Strongyloides. The highest faecal worm egg count was observed at d 1 p.p. ($P > 0.05$) only in the control ewes, however, huge variations were observed between the animals during this time point. Afterwards, constant values were observed in both groups. Nevertheless, neither the time nor Mg supplementation has effect on the faecal worm egg counts (Fig. 5-A).

During the ante-partum period (d 14 a.p.) ewes offered more Mg showed higher eosinophil count compared to the control ewes (Mg supplementation x Time interaction, $P < 0.001$), whereas the control ewes showed the highest numbers during the post-partum period ($P < 0.05$). At d 1 p.p. and d 30 p.p. a decline in the eosinophils numbers was observed in both groups (Fig. 5-B). Additionally, no correlations were observed between the faecal egg counts and blood eosinophil numbers during the ante partum and the post partum periods in both groups.

Serum mineral and cortisol levels

Data on mineral and cortisol levels were previously submitted elsewhere. Briefly, in both groups, neither the time nor the treatment had a significant effect on the total Mg serum levels. Control ewes showed a significant decrease ($P < 0.05$) in the total Ca serum levels at d 1 p.p. compared to d 30 a.p. In addition, at d 14 a.p. Ca/Mg ratios were lower compared to d 30 a.p. and d 14 p.p. ($P < 0.05$). Ca and Ca/Mg values did not show differences throughout the TP in Mg ewes. Neither the time nor the Mg supplementation had significant effects on the serum cortisol levels throughout the TP. The obtained values showed huge variations among the animals. In both groups, the lowest values were reported at d 14 a.p., while the highest values were recorded at d 30 p.p. in the control group, and at d 14 p.p. in the Mg group.

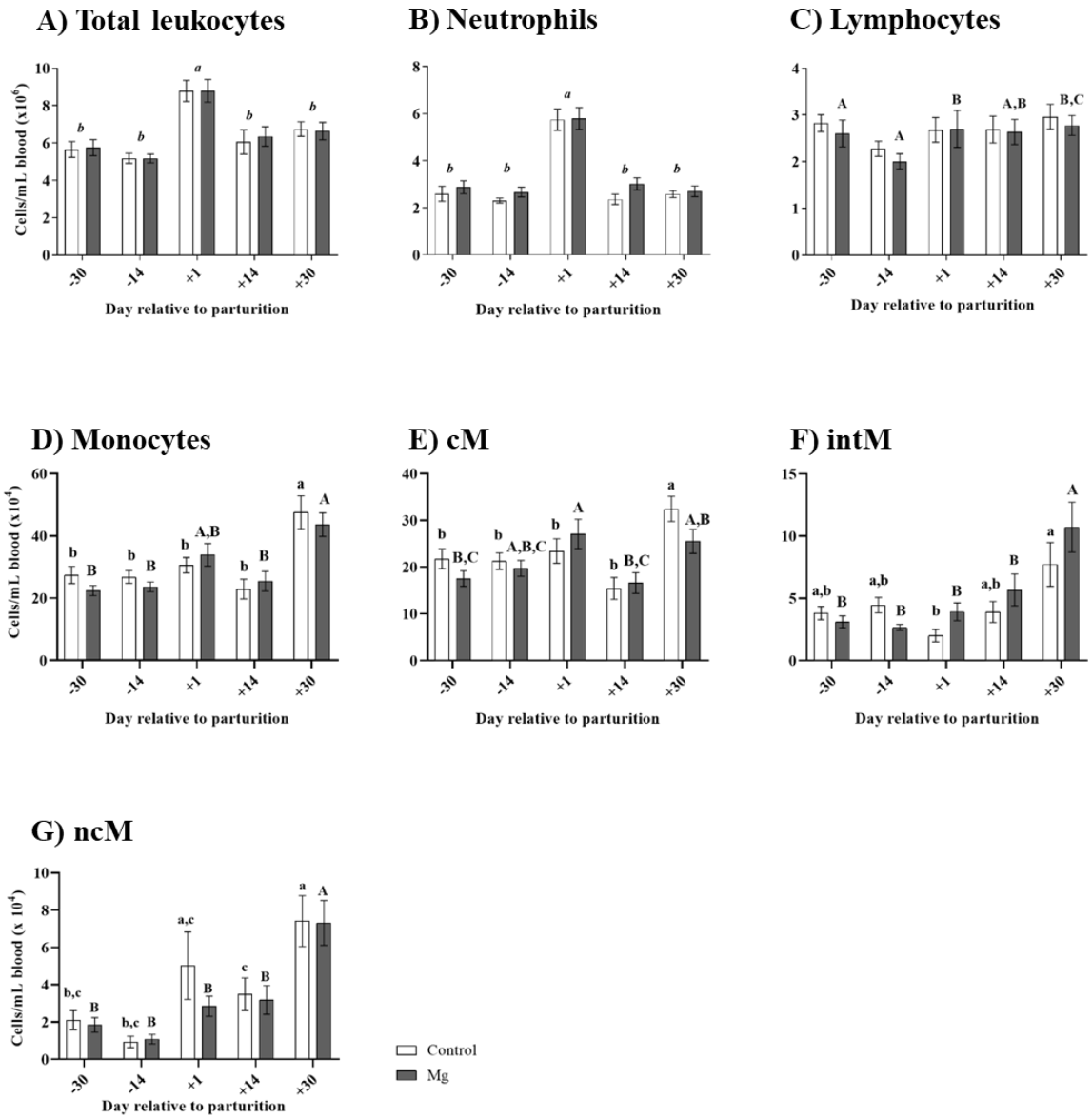

Fig. 1. Total leukocyte, neutrophil, lymphocyte, monocyte and monocyte subsets counts in ewes during the transition period and the effect of magnesium supplementation. The two-way ANOVA test and Sidak multiple comparisons test were used for comparison between the different time points and groups. Significant time-dependent differences ($P \leq 0.05$) are indicated by different small letters for the control group, capital letters for the Mg group and small italic letters for both groups. Mean ± SEM, (control group n = 9, Mg group n = 10).

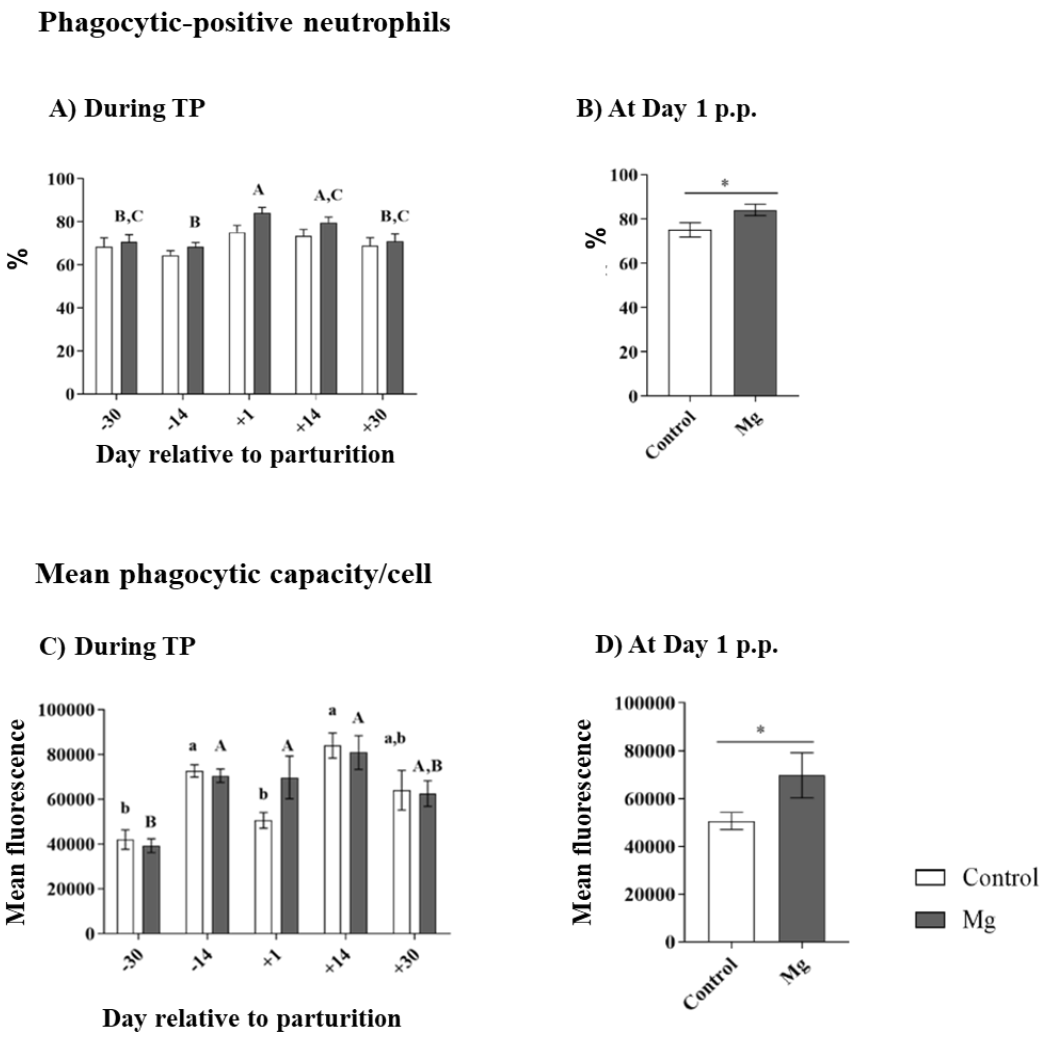

Fig. 2. Neutrophil phagocytic activity *in vitro* (% phagocytic positive neutrophil and mean phagocytic capacity/cell) in ewes during the transition period and the effect of magnesium supplementation. The unpaired t-test, two-way ANOVA test and Sidak multiple comparisons test were used for comparison between the different time points and groups. Significant time-dependent differences ($P \leq 0.05$) are indicated by different small letters for the control group and capital letters for the Mg group. Mean ± SEM, (control group n = 9, Mg group n = 10). Significant differences between the two groups are indicated as * ($P < 0.05$).

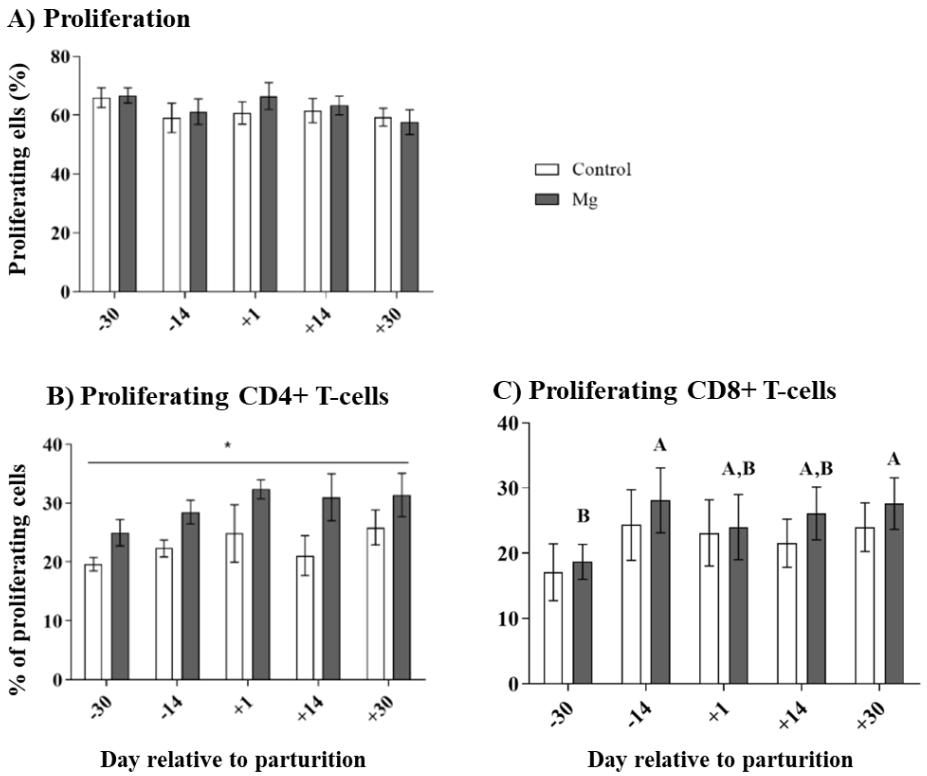

Fig. 3. T Lymphocytes proliferative capacity as well as CD4+ and CD8+ T cells response *in vitro* in ewes during the transition period and the effect of magnesium supplementation. The two-way ANOVA test and Sidak multiple comparisons test were used for comparison between the different time points and groups. Significant time-dependent differences ($P \leq 0.05$) are indicated by capital letters in the Mg group. Mean ± SEM, (control group n = 9, Mg group n = 10). Significant differences between the two groups are indicated as *($P<0.05$).

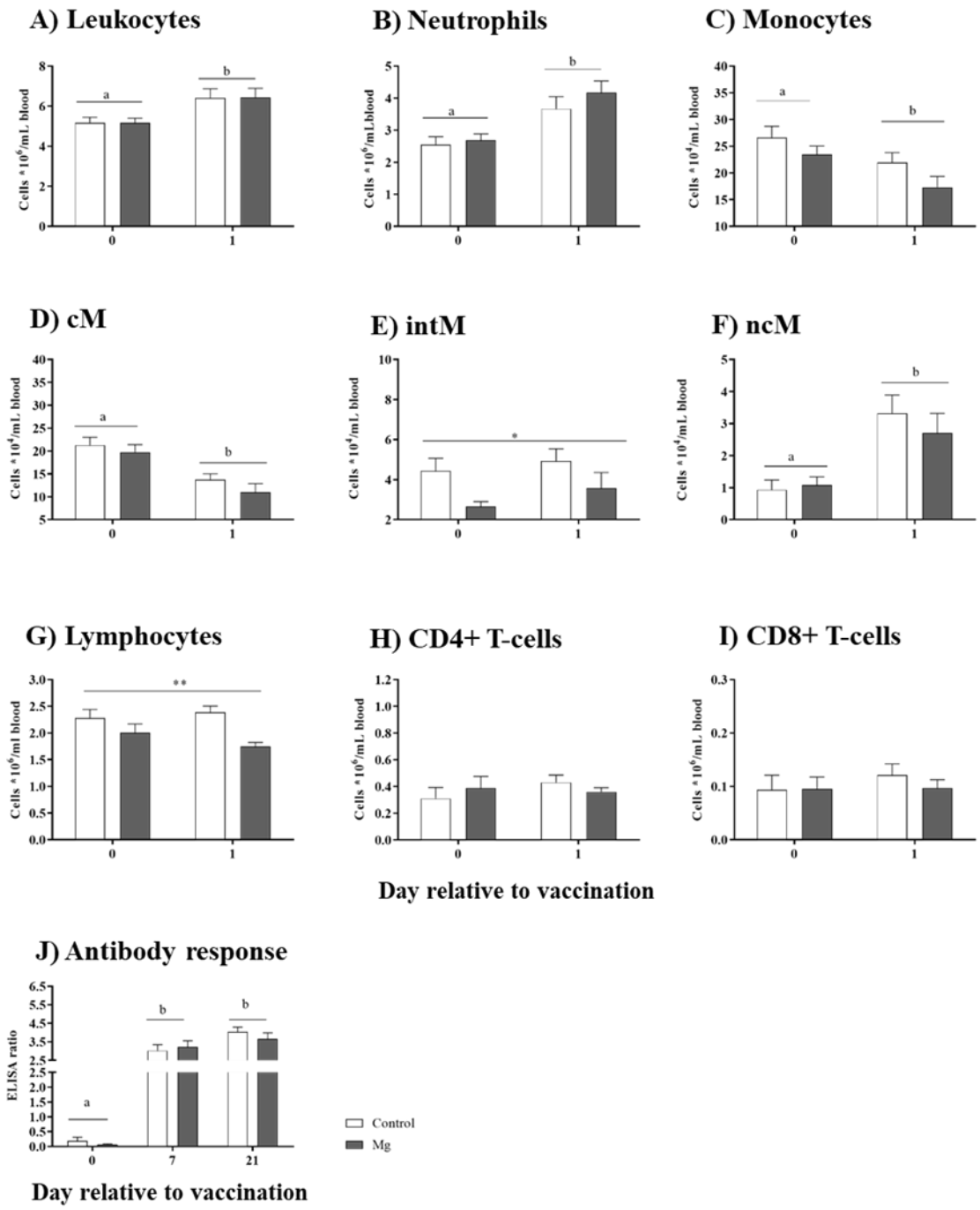

Fig. 4. *Mycobacterium avium paratuberculosis* MAP vaccination induced cellular and humoral response in ewes during the transition period. Ewes were vaccinated at d 14 a.p, blood samples were collected before the vaccination (0) and 24 h following the vaccination (1) to investigate the cellular compositions (A, B, C, D, E, F, G, H and I), whereas serum samples were collected at d 7 and d 21 postvaccination to assess the MAP Abs response (J). The two-way ANOVA test and Sidak multiple comparisons test were used to detect the differences between control (n = 9) and Mg (n = 10) groups as well as the time points. Significant time-dependent differences ($P \leq 0.05$) are indicated by

different letters. Significant differences between the two groups are indicated as * (P < 0.05) and ** (P < 0.01). Mean ± SEM.

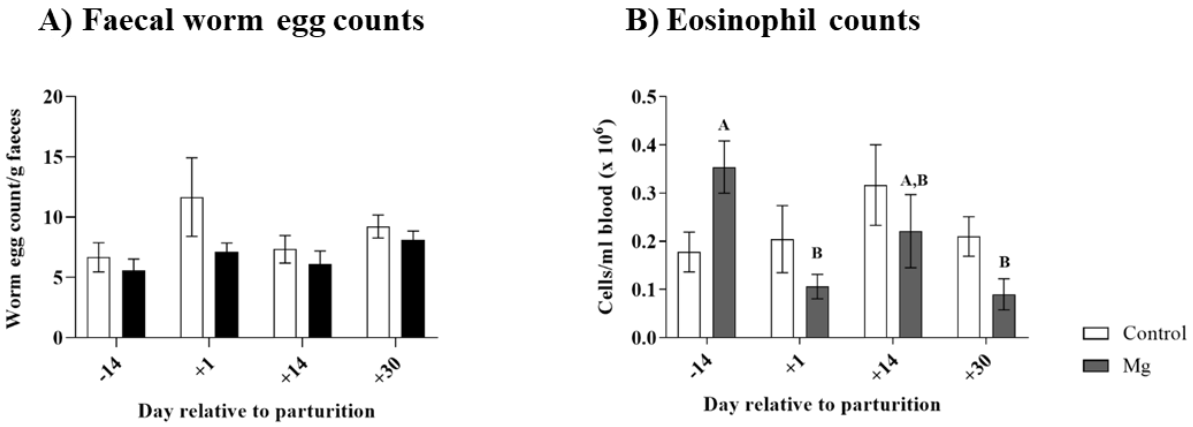

Fig. 5. Faecal worm egg counts (A) and blood eosinophils numbers (B) in ewes during the transition period and the effect of magnesium supplementation. The two-way ANOVA test and Sidak multiple comparisons test were used for comparison between the different time points and groups. Mean ± SEM, (control group n = 9, Mg group n = 10).

Discussion

Substantial changes are observed in the metabolic and endocrine profile as well as in the immune response in farm animals during the TP [1,2] concurrent with a high incidence of infectious diseases. Accordingly, the current study aimed to analyse changes in selected immune parameters in ewes during the TP and whether dietary Mg supplementation has an impact on these parameters. Based on the values of mineral concentrations and blood cell numbers reported in the present study the ewes were in normal physiological conditions [30].

Regarding the fluctuations in the circulating immune cells during the TP, blood cell numbers obtained in the present study behaved differently throughout the whole period. Total leukocytes and neutrophils reached a maximum at d 1 p.p. in both groups, similar results previously being observed in ewes [9, 31] and cows [6, 32]. The reason for neutrophilia around parturition is commonly due to high cortisol levels at this time, which contributes to downregulation of surface adhesion molecules expression [33] and possibly to up regulation of anti-adhesion molecules [34, 35], in addition to an enhanced release of cells from the bone marrow [37]. However, we did not observe a cortisol peak at

parturition as has been previously reported in cows [36, 37]. Moreover, cortisol values obtained in the present study throughout the TP were substantially higher than those previously reported for sheep [38, 39], which might have been due to capturing and fixation of the animals for blood sampling.

Ovine blood monocyte numbers peaked later at d 30 p.p. This kind of fluctuation in monocyte cell numbers during the TP was true for all identified monocyte subpopulations, which is in contrast to respective findings in cows [6] where these cells peaked together with neutrophils at parturition [6]. This might be reflected by the fact that that ovine monocytes display a different redistribution pattern during the TP compared to bovine counterparts. The obtained results showed high similarity in the classification system for ovine monocyte subsets with human and bovine classical, intermediate and non-classical monocytes [40, 41] and the results are comparable to previous ovine studies [42, 43]. However, to the best of our knowledge this is the first study to investigate the kinetics of ovine blood monocyte subsets during the TP.

Blood lymphocyte numbers showed the lowest values before parturition and then increased at parturition which is in line with previous study in cows [44], whereas stable numbers throughout the TP were reported by Eger et al, [6] and Cui et al, [26].

Overall, ovine blood neutrophil and lymphocyte numbers during the TP showed the same pattern as in cows whereas the monocytes and their subsets behaved differently in periparturient ewes. Notably, the supplementation with Mg did not alter any of the cell-type-specific redistribution pattern.

During late pregnancy and early lactation ewes generally experience a rise in faecal worm egg counts (Periparturient rise (PPR)) [9], which is associated with a temporary decrease in host immunity as reported by reduction in the circulating eosinophil numbers and antibody levels against specific nematodes [9]. In the present study, we weren't able to detect a classical PPR, maybe due to high variation in faecal egg count among the animals, however, the highest faecal worm egg count was reported at d 1 p.p. in the control ewes, a larger group size might be needed to detect significant differences. In parallel blood samples were collected to determine the blood eosinophil numbers, the highest value was observed at d 14 p.p. in the Mg ewes which point towards an interaction between Mg supplementation and time, whereas the lowest values were observed at d 1 p.p. and d 30 p.p. in the both groups, our findings regarding the decline in eosinophil numbers at parturition are in line with a previous study [9]. A negative association between blood eosinophil numbers and faecal worm egg counts was reported previously in ewes during

late pregnancy and parturition as well as early lactation periods [9] which could point towards a link between PPR and broader tissue changes (parturition and lactation) associated with relaxation or redirection of immune responsiveness. Further research is needed to investigate the interaction between Mg supplementation and PPR phenomenon in ewes.

Parturition is associated with a remarkable alteration in nutrient and metabolic demand as well as hormonal changes [45, 46], which can be associated with impaired immune function [47, 48]. Several studies reported on neutrophil dysfunctions during the peripartum period, such as a reduction in superoxide production, phagocytosis, the expression of cell surface adhesion molecules, and the capacity to migrate [36, 49].

In the present study, the percentage of phagocytosis-positive ovine neutrophils did not change substantially during the TP with lower values during the ante-partum period. However, the individual cell capacity for phagocytosis changed more drastically, with highest values at d 14. p.p.. This change in the phagocytic capacity of ovine neutrophils during the TP is in line with a study in cows [45], while it contrasts to the data demonstrated by Batistel et al. [50], who reported lowest phagocytosis values at d 1 p.p..

In the present study, at d 1 p.p., ewes supplemented with Mg showed both a significantly higher percentage of phagocytosis-positive neutrophils, as well as a higher phagocytic capacity per cell compared to the control ewes. Comparable studies are scarce. Our findings are supported partly by *in vitro* data obtained with a monocyte-like human lymphoma U937 cell line where extracellular Mg levels correlated positively with an enhanced phagocytosis rate [51]. The enhancement in the phagocytic potential of neutrophils could be due to the enhancement in glucose viability and cellular uptake [18, 52]. Noteworthy, glucose levels showed less fluctuation throughout the TP in Mg supplemented ewes compared to the control group (data not shown). Moreover, ewes offered more Mg showed lower glucose levels compared to the control group at d 1 p.p., which could be linked to higher insulin sensitivity and increased glucose disposal in Mg supplemented ewes.

We extended the analysis of Mg supplementation effects on immune functions to the proliferative capacity of lymphocytes. This function appeared to be rather stable throughout the TP when considering the proliferation of all lymphocytes with no apparent influence of Mg supplementation. These findings were in parallel with Mg-supplemented rats whose *in vitro* ConA-simulated splenocytes proliferated in a comparable way as cells in a control group [53].

From a physiological point of view, the stable pattern of lymphocyte proliferation obtained in the present study was in contrast to the bovine system where the proliferative capacity of lymphocytes was significantly lower around parturition [45] and increased gradually during the postpartum period [54, 55] . In cows, the reduction in proliferative responses around parturition was linked to high cortisol levels during this period [37]. The lack of such cortisol fluctuations in the ewes in our study may partially explain the differences to the bovine system.

The analysis of proliferating T lymphocyte subpopulations revealed some interesting details. In comparison to the control group, the proliferative response of CD4+ T cells in Mg-supplemented ewes was significantly higher throughout the whole TP. In contrast, the effects of Mg supplementation were less prominent on the proliferative capacity of CD8+ T cells, pointing towards a specific role of Mg for T-cell subpopulations. The enhanced CD4+ T cell proliferation could be due to the improvement in glucose viability and cellular uptake [18, 52], as the activation of cellular proliferation is initiated by growth factors that increase glucose uptake and protein synthesis [15]. The role of Mg in lymphocyte signalling pathway activation has been previously reported in human patients diagnosed with a mutation in an Mg transporter gene MAGT1 (novel X-linked human immunodeficiency). This condition is characterised by hypomagnesaemia, CD4+ lymphopenia and defective T-lymphocyte activation [56]. Knockout of another Mg transporter TRPM7 in chicken lymphocyte cell line stopped the lymphocyte proliferation activity *in vitro*, however, when Mg was added to the culture medium the cells resumed their proliferation [57]. Where Mg plays a role in the activation of T-lymphocytes is still a matter of debate. Mg may be involved in T cell receptor activation [56], the glycolysis process [57], or in the activation-induced Ca influx which partially depends on Mg [56].

Apparently, oral Mg supplementation displayed a modulatory role for ovine neutrophil and CD4+ T cell function measured ex vivo. The question whether Mg supplementation also affects complex immune responses depending on the complex interplay of soluble mediators and different cell types *in vivo* was addressed by analysing the immediate cellular response within 24 h following vaccination and the humoral immune response against *Mycobacterium avium subsp. paratuberculosis* (MAP) at days 7 and 21 post vaccination. These were rather early time points, since the follow up analysis of MAP vaccinations in the field includes the examination of subcutaneous nodules at the inoculation site between 15 to 30 days post vaccination [58], or determination of the IFN-α serum concentration and MAP-specific antibody levels within one or two months post

vaccination [58, 59]. In the present study, the vaccination resulted in a significant increase in MAP-specific antibody levels at d 7 and d 21 postvaccination. This rapid increase in IgG antibodies against MAP can be interpreted as a booster response of ewes, previously infected with MAP [60]. Since there were no significant differences in the antibody response between control and Mg supplemented ewes, this argues against an influence of Mg supplementation on the cascades leading to an activation of existing T and B memory cells.

Effects of vaccination could be observed much earlier (within 24 h): In both animal groups as blood total leukocyte, neutrophil and ncM numbers significantly increased whereas total numbers of blood monocyte and cM dropped post vaccination. The selective influence of Mg supplementation was apparent for blood intM and lymphocyte numbers in Mg supplemented ewes compared to the control ewes.

The observed changes in blood leukocyte subpopulation numbers are most likely due to distal effects of the spectrum of vaccination-induced mediators. Thus, differences between the control and the Mg-supplemented group might point towards an influence of dietary Mg on the regulated release of such factors from vaccine/adjuvant-triggered cells, e.g. dendritic cells as has been reported previously [61, 62]. To the best of our knowledge the influence of Mg on the early innate immune response following vaccination has not been addressed so far.

Conclusion

Transition period in ewes is associated with fluctuations in blood myeloid cell numbers, which are only partially comparable with those of cows. A significant depression in selected immune cell functions does not occur during the ovine TP. This species-specific circulatory behaviour and function of immune cells during the TP should be considered when ewes are used as model animals. Dietary Mg supplementation does not interfere to a large extent with the circulation behavior of immune cells. In fact, it selectively favours the functional capacity of neutrophils and T-lymphocyte subsets during the TP. The impact of dietary Mg on the composition of circulating immune cells after vaccination against MAP suggests that Mg modulates early vaccine-induced innate immune mechanisms. Whether this holds true for different vaccines needs further analysis.

Methods

Animals and feeding regimes

Animals: Twenty-three German Blackhead Mutton ewes (entering the 2nd and 3rd lactation, age: 2-3 years, weight: 85-100 kg) were synchronized with Chronogest CR intravaginal sponges (20 mg, flugestone acetate, MSD Santé Animale, Beaucouzé, Cedex, France), and mated naturally with three rams from the same breed. At day 39 post-mating, ewes were transabdominal scanned for pregnancy by using a diagnostic scanner, out of the twenty-three ewes, nineteen became pregnant.

The nineteen pregnant ewes were randomly divided into two groups: control group (n = 9) and Mg group (n = 10). The ewes were housed in the facilities of the Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Mecklenhorst, Germany.

Feeding regimen: In brief, the animals were group-fed under surveillance of the personnel. Throughout the experimental period, there were no significant refusal. The ewes received grass silage (3 kg per ewe and day, 32% dry matter, DM) and concentrate (500 g per ewe and day, 89% DM). While the control group was fed a common concentrate available for sheep (Raiffeisen Schafe S2 lose, Agravis Niedersachsen-Süd GmbH, Hannover, Germany) containing 0.29% Mg (as fed), the pellets for the Mg group had been additionally supplemented with magnesium oxide to a final content of 0.51% (as fed). After parturition (48 h post-partum), the amount of concentrate was increased to 1200 g per ewe and day (as fed). Ante-partum, the estimated daily Mg intake was 2.97 g per ewe in the control group (0.21% of DM) and 4.19 g per ewe in the Mg group (0.30% of DM). Postpartum, the estimated daily Mg intake was 4.96 g per ewe in the control group (0.24% of DM) and 7.74 g per ewe in the Mg group (0.38% of DM). Ewes were allowed to adjust to this diet for two weeks before starting the experiment (adaptation period). The groups were housed separately with water available *ad libitum*. No antihelmintic treatment was applied for these animals during the experimental period.

Blood collection

Blood was obtained by jugular vein puncture into K_2E (EDTA), sodium heparin, and Clot Activator Tube (CAT) vacutainer tubes (BD Vacutainer systems, Roborough**,** UK) at five time points: d 30 a.p., d 14 a.p., d 1 p.p., d 14 p.p. and d 30 p.p. at 08:00 before the morning feeding. Serum was separated by centrifugation of clotted blood (3000 g, 10 min, 4 °C) and stored in aliquots at – 20 °C for further analysis.

Serum variables

Total serum calcium levels were measured using a commercial kit (Labor + Technik LT-SYS, Labor + Technik Eberhard Lehmann GmbH, Berlin, Germany) spectrophotometrically (Uvikon XL UV-Visible, Scanning spectrophotometer, Biotek Instruments Inc., Winooski, VT, USA). Total Mg, was determined by using commercial kits (Labor + Technik LT-SYS, Labor + Technik Eberhard Lehmann GmbH) in the diagnostic laboratory of the Clinic for Swine, Small Ruminants and Forensic Medicine, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. Serum cortisol levels were estimated using ABNOVA® Sheep cortisol ELISA kits (Abnova, Taoyuan, Taiwan) in accordance with the manufacturer's instructions.

Vaccination

At d 14 a.p., the ewes in both groups were injected s/c with 1 mL of a commercial vaccine against *Mycobacterium avium paratuberculosis* (MAP), strain 316 F, (Gudair®, CZ Veterinaria, S.A, Pontevedra, Spain). Whole blood samples were collected immediately before the injection (0) and 24 h following the vaccination (1), to assess vaccination-associated changes in the composition of blood leukocytes (neutrophils, lymphocytes, CD4+ and CD8+ cells, monocytes, and monocyte subsets) see below. At d 0, d 7 and d 21 post vaccination serum samples were taken and transferred to the diagnostic laboratory of the Clinic for Swine, Small Ruminants and Forensic Medicine University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, to determine the level of MAP-specific antibodies (MAP Abs) using a commercial diagnostic indirect ELISA (Cattletype® MAP Ab, Indical bioscience, Leipzig GmbH, Germany) in accordance with the manufacturer's instructions. Results are given in optical densities (OD). Mean values (MV) of the ODs for the negative (NC) and the positive Control (PC) were calculated. The ratio of sample OD to mean OD of the positive control (S/P) was calculated according to the following equation:

$$\frac{S}{P} = OD\ Sample - MV\ OD\ NC\ \div MV\ OD\ PC - MN\ OD\ NC$$

As suggested by the manufacturer, an S/P ratio of ≥ 0.4 was considered positive.

Total leukocyte counts

Whole heparinised blood (50 µL) was mixed with 450 µL Turk´s solution, and 20 µL were applied to a Neubauer chamber. Leukocytes were counted microscopically (Nikon microscope ECLIPSE 80i). Fractions of neutrophils, lymphocytes and monocytes among blood leukocytes were determined flow cytometrically (Fig. S1). For this purpose, 100 µL whole heparinised blood was mixed with 500 µL distilled water (DW) for 3 s, followed by addition of 500 µL double concentrated phosphate buffered saline (2xPBS). After centrifugation (517 x g, 4 min, 8 °C), the supernatant was discharged and the cell pellet was resuspended in PBS. In the case of the remaining erythrocytes, the hypotonic lysis step was repeated until complete erythrolysis. The last cell pellet was suspended in 100 µL PBS (2 µg/mL propidium iodide) and the cell suspension was measured by flow cytometry (BD Accuri C6 flow cytometer®, Becton Dickinson Inc., Holdrege, NE, USA). For each sample 20,000 events were collected. After setting regions to identify propidium iodide-negative (viable) cells (Fig.S1-A), identification of singlets among viable leukocytes was performed in a forward scatter area (FSC-A) versus FSC-height density plot A (Fig.S1-B). Afterwards, granulocytes (neutrophils), lymphocytes and monocytes were determined among the singlets based on their characteristic forward scatter area (FSC-A) and side scatter area (SSC-A) properties (Fig.S1-C). The percentages of the major leukocyte subpopulations (lymphocytes, monocytes and granulocytes) were determined. The obtained percentages were multiplied with the total leukocyte counts to obtain total numbers of these cell types among leukocytes.

Neutrophil phagocytic activity in whole blood samples

In vitro phagocytosis was performed as previously described [65] with some modifications. Fresh heparinised whole blood (100 µL) was incubated with heat-killed, FITC-labelled *Staphylococcus aureus* (0.5×10^9 bacteria in 400 µL PBS) (Institute of Microbiology, University of Veterinary Medicine, Hannover, Germany). In 1 mL vials; this bacteria suspension was added to 100 µL blood to achieve a bacteria/neutrophil ratio of 50:1. The needed volume of bacteria suspension was calculated based on total numbers of neutrophils/mL blood. Mixtures were incubated for 30 min (37 °C, 5% CO_2). Blood samples with added PBS (same volume as the bacteria suspension) served as controls. Thereafter, blood/bacteria mixtures were subjected to a hypotonic lysis step by adding 500 µL D.W for 3 s followed by adding 500 µL 2xPBS. The mixture was centrifuged (517 x g,

4 min, 8 °C) and the cell pellet was resuspended in 1 mL PBS (2 µg/ml PI). Neutrophil phagocytic activity was defined flow cytometrically as the percentage of green fluorescent granulocytes (cells were identified based on forward/side scatter characteristics) among viable (PI negative) granulocytes after excluding eosinophils on FITC-fluorescence vs SSC-A density plots (Fig. S2-A-D). The mean cellular phagocytic capacity was defined as the geometric mean green fluorescence of phagocytosis-positive (green fluorescing) granulocytes.

Gradient density separation of mononuclear cells

Separation of mononuclear cells was performed as described previously [41] with some modifications. A total of 20 mL fresh EDTA blood was diluted 1:1 PBS, layered gently on the top of 15 mL lymphocyte separation medium (Density: 1.077 g/mL, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and centrifuged (1000 x g, 30 min, 4 °C). The interphase containing mononuclear cells (MNC) was collected and placed in a fresh 50 mL tube. This was filled up to 50 mL with PBS and centrifuged (500 x g, 10 min at 4 °C). The supernatant was discharged and the pellet resuspended. Erythrocytes lysis step was performed by adding 20 mL DW, mixing it for 3 s and then adding 20 mL 2xPBS. After centrifuging (250 x g, 10 min at 4 °C) the supernatant was discharged and the pellet was resuspended in 25 mL PBS followed by centrifugation (120 x g, 10 min at 4 °C). The final cell pellet was resuspended and adjusted to $10x10^6$/mL in PBS. The purity of the MNC separation was determined flow cytometrically on an SSC-A vs FSC-A density plot (S3-A).

Monocyte subset determination

Separated MNC were placed in a 96 well round bottom plate (2×10^6 MNC /well) and 30 µL of a mixture of two directly conjugated, ovine cross-reactive, monoclonal mouse anti-human antibodies was added: anti-CD14-RPE (BIO-RAD, MCA1568PE, 100 TESTS/mL, final dilution 1:10), anti-CD16-FITC (BIO-RAD, MCA5665F, 0.1 mg/mL, final dilution 1:45). The mixture was incubated for 30 min at 4 °C. Cells were washed twice with 200 µL membrane immunofluorescence (MIF) buffer (PBS + 2.5g/L bovine serum albumin + 0.1 g/L of NaN_3). Afterwards, 100 µL PI was added to exclude the dead cells, after gating MNC according to their FSC-A and SSC-A properties (Fig. 3S-A,B,C). Three ovine monocyte subsets were defined flow cytometrically based on their CD14 and CD16 expression: classical monocytes (cM, CD14+/CD16-), intermediate monocytes (intM,

CD14+/CD16+), and nonclassical monocytes (ncM, CD14-/CD16+) (Fig. 3S-D). Preliminary double staining of cells with concentration-matched isotype controls (IgG2a-PE, BIO-RAD MCA929PE, IgG2a-FITC, BIO-RAD MCA929F) ensured that murine IgG2a antibodies display no unspecific reactivity with sheep monocytes. Total counts of monocyte subsets were calculated by multiplying absolute monocyte counts with monocyte subset percentages obtained after flow cytometric analysis.

Lymphocyte proliferation capacity

Separated MNCs (10 x 10^6/mL) were labelled with carboxyfluorescein succinimidyl ester (CFSE, 1.5 µg/mL in BPS) (C1157, ThermoFisher Scientific In., Waltham, MA, USA) and incubated for 10 min at 37 °C. The double volume of culture medium (RPMI 1640 medium, SIGMA-Aldrich®, Darmstadt, Germany) supplemented with 10% foetal calf serum (Biochrom AG, Berlin, Germany), and 100 U/mL Penicillin/Streptomycin (Invitrogen GmbH, Karlsruhe, Germany) was added and the cell suspension was centrifuged (500 x g, 10 min at 4ºC). The supernatant was discharged, 50 mL PBS was added and the suspension was centrifuged again (500 x g, 10 min at 4 ˚C). The last cell pellet was resuspended in 4 mL culture medium and adjusted to 3 x 10^6/mL. Afterwards, the CFSE-labelled MNCs (3×10^5 cells/well) were stimulated with Concanavalin A (ConA, 4 µg/mL, Sigma-Aldrich, Biochemie GmbH, Hamburg, Germany) in 96-well round bottom plates. Each setup was done in duplicate. Set ups without ConA served as controls. Plates were incubated for 4 days (37 ºC, 5% CO_2 in air). Thereafter, the plates were centrifuged (351 x g, 4 min, 8 °C), the supernatant was removed and the cells were incubated with directly labelled with a murine monoclonal antibody specific for sheep CD4 (anti sheep CD4-Alexa Fluor®-647, BIO-RAD MCA2213A647, 1:160) and a monoclonal antibody cross reactive with sheep CD8 (anti bovine CD8-RPE, BIO-RAD MCA837PE, 1:40). Preliminary staining of cells with concentration-matched isotype controls (IgG2a-PE, BIO-RAD MCA929PE, IgG2a-Alexa-Fluor-647, BIO-RAD MCA MCA929A647) ensured that murine IgG2a antibodies display no unspecific reactivity with sheep lymphocytes. After a 30 min incubation period at 4 °C cells were washed twice with MIF buffer as described above and resuspended in MIF buffer containing 2 µg/ml PI. Labelled cells were analysed flow cytometrically. CFSE fluorescence of viable (PI-negative) mononuclear cells was plotted against the cell size (FSC-A) (Fig. S4-A, B). Cells displaying reduced CFSE fluorescence were identified as activated/proliferating cells. (Fig. 4S-C). The proliferative capacity of T-cell subsets was

determined in CFSE versus CD4-Alexa 647 and CFSE versus CD8-PE density plots, respectively (Fig. S4-D, E).

Faecal worm eggs count

Rectal faecal samples (10-15 g) were collected from individual animals at d 14 a.p, 1 p.p., 14 p.p and 30 p.p., and transported to the diagnostic laboratory of the Clinic for Swine, Small Ruminants and Forensic Medicine, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany.

Faecal samples were examined using saturated Nacl as a flotation method [66]. With this method roundworms were differentiated microscopically according to the size and shape of the eggs. The roundworm species with eggs of the same shape (e.g. *Haemonchus contortus, Teladorsagisa circumcinta, Trichostrongylus colubriformis* and others) were not differentiated.

Briefly, 10 g faecal samples were suspended in D.W, sieved through a grid into a beaker in a volume of 250 ml and allowed to stand for 30 min. 2 mL of the sediment was resuspended in 9 mL saturated Nacl and centrifuged (90 x g/3 min). Three drops were taken from the surface of the liquid and placed in glass slide which covered with cover slip and examined under a microscope (10X), results were expressed as eggs per gram.

Sodium silicate solution was used for sedimentation of egg of liver fluke worms (*Fasciola spp.*). For this method 2 mL of the sediment was resuspended in 9 mL sodium silicate, 200 µL of methylene blue 3% were added and mixed well, afterwards the tubes were centrifuged (800 x g/10min). Three drops were taken from the surface of the liquid and placed in glass slide which then was covered with cover slip and examined under the microscope (10x). The results of this investigation were negative, since eggs of liver fluke worms were not found.

Eosinophils count

Blood was obtained by jugular vein puncture into K_2E (EDTA) vacutainer tubes (BD Vacutainer, Belliver Industrial Estate, Plymouth, UK) in parallel with the collection of the faecal samples. The percentage of lymphocytes, neutrophils, monocytes and eosinophils were determined microscopically (counting 200 leukocytes in thin Giemsa May-Grunwald-stained blood smears). Eosinophils total counts were calculated by multiplying the percentage of eosinophils among leukocytes with the total numbers of leukocytes determined whith a hematology analyser (Celltac Alpha Nihon Kohden Europe GmbH, Rosbach vor der Höhe, Germany).

Flow cytometric data evaluation

After acquisition, flow cytometric data were analysed with the Acurri BD^{TM} C6 software. The gating strategies to identify cell populations, frequencies of phagocytosis-positive cells, proliferating cells, CD4+ and CD8+ positive cells among proliferating cells, and mean fluorescence intensities of phagocytosis-positive cells are described in supplemtatry figures S1 to S4.

Statistical analysis

The data were expressed as mean ± SEM, n representing the number of animals per group. The unpaired t-test, two-way ANOVA test and Sidak multiple comparisons test (GraphPad Prism 8 Software, San Diego, CA, USA) were used for comparison between the different time points and groups. Correlations between selected variables (faecal worm egg counts and eosinophil numbers) were analyzed by Pearson's correlation. Differences were considered statistically significant when $P < 0.05$. All data except faecal egg counts were normally distributed according to Shapiro-Wilk and Kolmogorov-Smirnov tests. Log-transformed faecal egg counts were normally distributed. Two-way ANOVA analysis of faecal egg counts was performed with antilogarithmic values calculated with the EXP function (Microsoft Excel).

Abbreviations

CD: Cluster of differentiation; CFSE: carboxyfluorescein succinimidyl ester; cM: Classical monocyte; ConA: Concanavalin A; FITC: Fluorescein isothiocyanate; FSC-A: forward scatter area; GMB: German black headed mutton; intM: Intermediate monocyte; MAGT1: magnesium transporter 1; MAP: Mycobacterium avium paratuberculosis; Mg: Magnesium; MIF: membrane immunofluorescence; MNC: mononuclear cells; ncM: Nonclassical monocyte; PI: propidium iodide; PPR: Periparturient rise; SSC-A: Side scatter area; TP: Transition period; TRPM7: transient receptor potential melastatin family member 7

Declarations

Ethics approval and consent to participate

This study was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (33.19-42502-05-18A359). A written consent to use the animals was obtained from the Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute

(FLI), Mecklenhorst, Germany. All procedures involving animals were carried out in accordance with German legislation on animal welfare.

Consent for publication

Not applicable.

Availability of data and materials

Data sets generated from this study are available upon request to the corresponding author.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Conceived and designed the experiments: GB, HJS, MG, MRW and MHA. Performed the experiments: MHA. Analysed the data: MHA. Wrote the manuscript: MHA and HJS. All authors have read and approved the manuscript.

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Supplementary figures

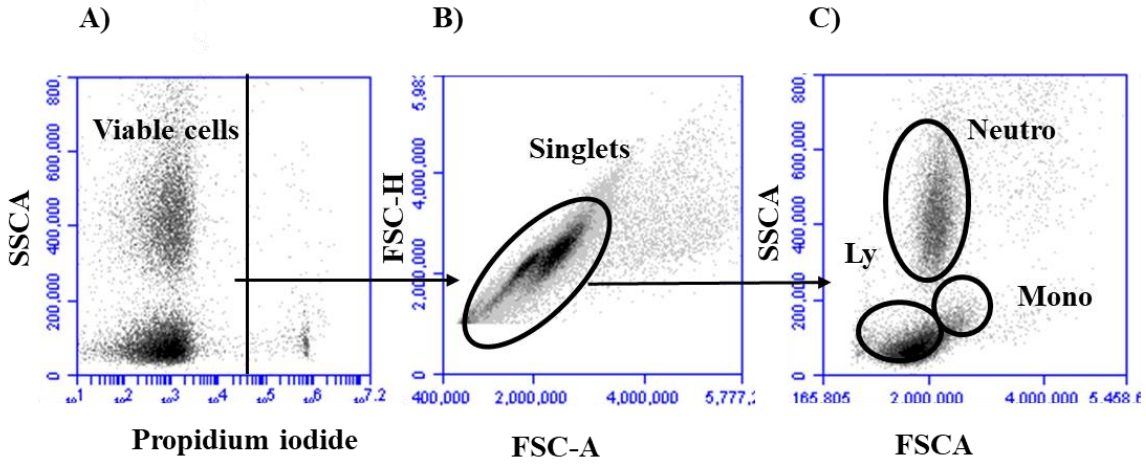

Fig. S1. Flow cytometric determination of ovine leukocyte composition. **(A)** Viable, propidium iodide-negative leukocytes after hypotonic lysis of heparinised blood were identified in a propidium iodide versus side scatter density plot. **(B)** Identification of singlets among viable leukocytes in an FSC-area versus FSC-height density plot. **(C)** Leukocytes gated on viable and single cells were plotted in FSC-A vs SSC-A density plot. Neutrophils (Neutro), lymphocytes [66], and monocytes (Mono) were identified based on their characteristic size (FSC) and complexity (SSC). Representative data from one animal.

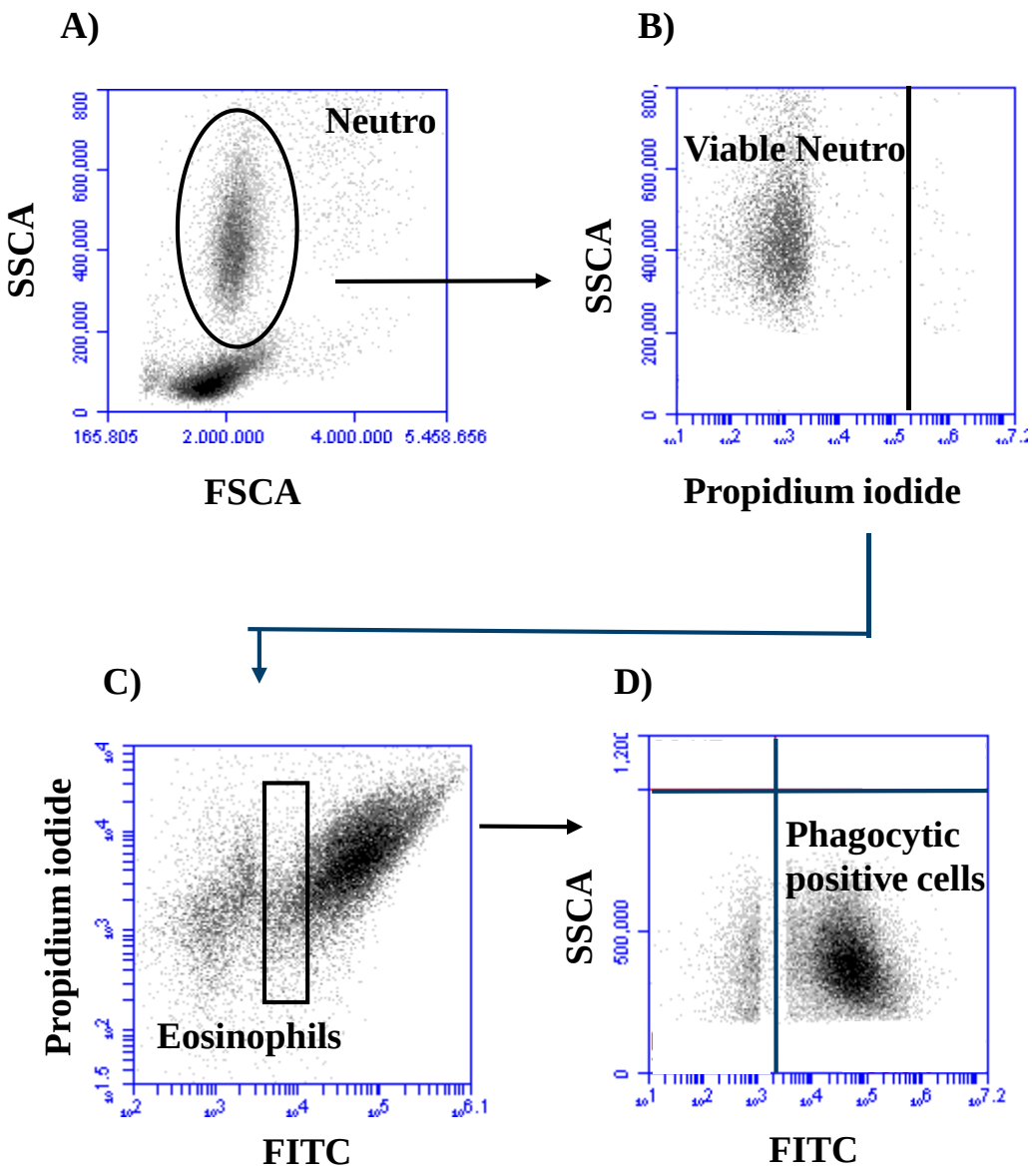

Fig. S2. Flow cytometric determination of ovine neutrophil phagocytic activity *in vitro*. Heparinized blood was incubated with or without heat-killed FITC-labelled *S. aureus* (Cells:Bacteria = 1:50). **(A)** FSCA vs SSCA density plot of leukocytes after hypotonic lysis and identification of neutrophil (Neutro). **(B)** Identification of viable, propidium iodide-negative neutrophil. (**C)** Identification of eosinophils in an FITC versus propidium iodide density plot. For assessing neutrophil phagocytosis in control set ups and samples with FITC-labelled *S. aureus* **(D)** in FITC versus SSC-A density plots, eosinophils were excluded from the analysis. Phagocytosis activity was defined as the percentage of green fluorescent cells among viable neutrophil (**D** lower right quadrant). Representative data from one animal.

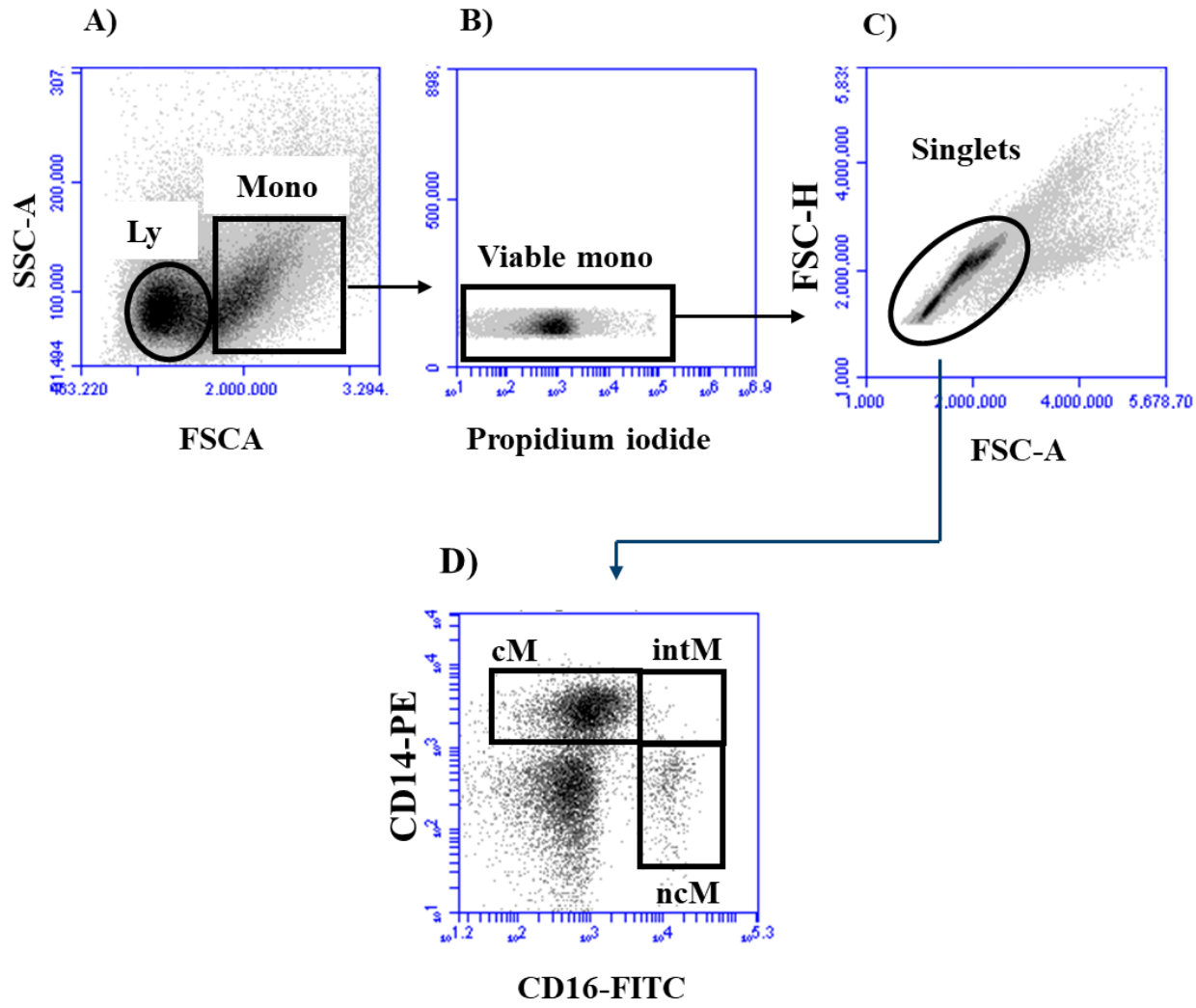

Fig. S3 Flow cytometric determination of ovine monocyte subpopulations. **A**) Monocytes (Mono) were identified among blood mononuclear cells based on their size and complexity. **B**) Identification of viable, propidium-negative Mono and (**C**) identification of single cells among viable Mono. **D**) Correlated density plot of Mono stained with directly labelled monoclonal antibodies specific for CD16 and CD14 and identification of classical (cM, CD14 ++ CD16-), intermediate (intM, CD14 ++ CD16+) and non-classical monocytes (ncM, CD14 - CD16++). Representative data from one animal.

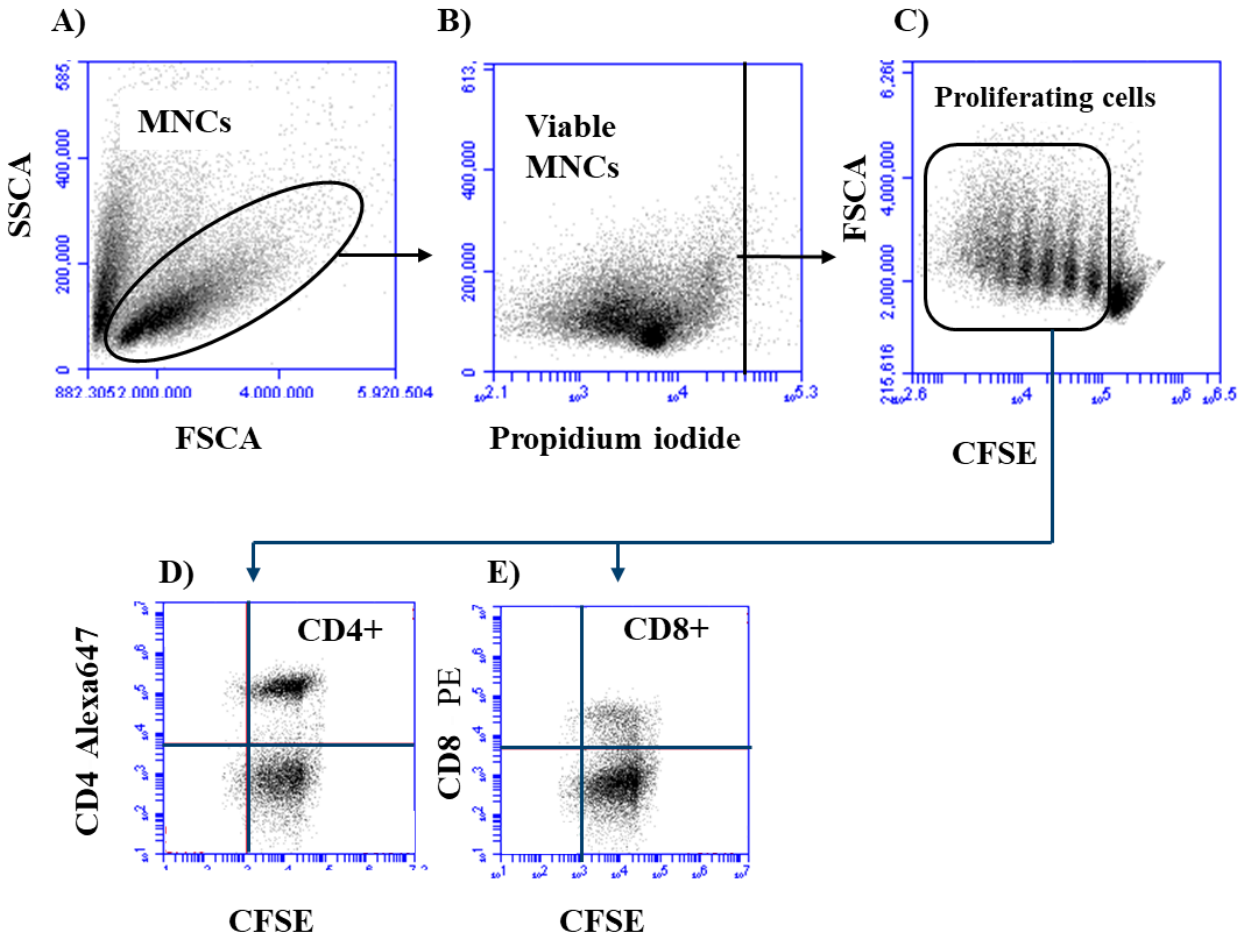

Fig. S4. Flow cytometric determination of ovine blood mononuclear cell proliferation *in vitro*. Ovine mononuclear cells were obtained after density gradient separation and labelled with CFSE (1.5 µM). Cells were stimulated with Con A (4 µg/mL) and incubated for four days at 37 °C *in vitro*. Set-ups without ConA served as a control. After incubation cells were labelled with antibodies specific for CD4 (Alexa- 647) and CD8 (PE) and analysed for morphology (identification of mononuclear cells in FSCA vs SSCA density plot, A), and viability (propidium-iodide-negative cells, B).The CFSE fluorescence of viable mononuclear cells was plotted against the cell size (FSCA) (C). Cells showing no reduction in CFSE fluorescence were identified as resting cells. Small cells and cells with reduced CSFE fluorescence were identified as proliferating cells) (C). The proliferative capacity of T-cell subsets was determined in CFSE versus CD4-Alexa 647 (D) and CFSE versus CD8-PE density plots (E). (representative data from one animal).

6 Manuscript 3

Expression of glucose and magnesium transporter genes in leukocytes of lactating ewes supplemented with magnesium

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Collection of the blood samples: Mona H. Ahmed, Mirja R. Wilkens

Laboratory work: Mona H. Ahmed, Gerhard Sponder

Statistic analysis: Mona H. Ahmed

Scientific writing: Mona H. Ahmed

Optimizing the manuscript: Gerhard Sponder, Jörg R. Aschenbach, Gerhard Breves

Expression of glucose and magnesium transporter genes in leukocytes of lactating ewes supplemented with magnesium

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Simple Summary: Transition from late pregnancy to early lactation is known as one of the most critical periods in the reproductive cycle of farm animals. This period is characterized by physiological and metabolic changes contributing to immunodysregulation. At cellular level magnesium (Mg) is involved as co-enzyme in more than 600 metabolic reactions associated with energy and glucose metabolism. Therefore, the main aim of the current study was to investigate the influence of dietary Mg supplementation on the expression of glucose and Mg transporter associated genes in leukocytes of lactating ewes. Our results showed that the expression of the variety of glucose and Mg transporters did not show any significant differences in response to dietary Mg supplementation. However, a tendency towards higher expression of glucose transporter 1 in the ewes offered more Mg might support an involvement of Mg in glucose metabolism of ovine leukocytes.

Abstract: Lactation is characterized by an increase in energy demand and glucose consumption, which may also be linked to the concurrent compromise in immune function during early lactation. Magnesium (Mg) could be involved in this link based on its role for more than 600 metabolic reactions associated with energy metabolism, in addition to its essential role in glucose metabolism. Therefore, the current study aimed to investigate the influence of Mg supplementation on the expression of glucose and Mg transport associated genes in leukocytes of lactating ewes, further, and to identify possible correlations between the expression of these genes and leukocyte subpopulations.

Pregnant ewes were divided into a control group (Control, n = 8) and a Mg group (Mg, n = 8), the later supplemented with magnesium oxide resulting in a daily Mg intake of approximately 0.30% and 0.38% of dry matter during the last four weeks of gestation and the first four weeks after lambing, respectively. At the end of this supplementation period blood samples were collected in PAXgene tubes in order to determine the mRNA expression of glucose transporters (GLUT1, 3 and 4) and Mg transporters (SLC41A1, CNNM2, TRPM6, TRPM7 and MagT1) in leukocytes.

Higher leukocyte expression of GLUT1 was detected as a tendency ($P < 0.087$) in the Mg group compared with the control ewes. Only numerical increases ($P > 0.05$) were observed in the expression of GLUT3, SLC41A1, CNNM2, TRPM6, TRPM7, and MagT1 in the ewes offered more Mg. Interestingly, GLUT4 was not expressed in either group. Numbers of circulation neutrophils, lymphocytes and monocytes showed no significant differences between the two groups. Furthermore, no correlations were observed between the expression of the investigated genes and certain leukocyte subpopulation numbers.

In conclusion, the expression of the variety of glucose and Mg transporters did not show any significant differences in response to dietary Mg supplementation. The tendency towards higher expression of GLUT1 in the Mg group might support an involvement of Mg in glucose metabolism of ovine leukocytes.

Keywords: magnesium; leukocytes; ewes; lactation; glucose; magnesium; transporters.

1. Introduction

In the mammalian system, magnesium (Mg) is the second most abundant intracellular cation and the fourth most abundant cation in the body. Physiologically, blood levels of total Mg concentration range between 0.7-1.15 mM in most species [1].

At the cellular level, Mg is a co-factor for more than 600 enzymes involved in energy metabolism, membrane functions such as various receptors, hormone-receptor binding, gating of calcium channels, transmembrane fluxes of cations and anions, regulation of adenylate cyclases, regulation of DNA and RNA molecular structure and repair mechanisms and control of intracellular Ca^{2+} release. In addition, Mg exhibits numerous structural attributes, it stabilizes cell membranes, regulates cell growth and reproduction [2].

Moreover, Mg is involved in activation of insulin receptors (phosphorylation of tyrosine kinase) and participates in glucose transport across cell membranes [3,4].

Numerous Mg transporting/homeostatic proteins are involved in cellular Mg homeostasis (Table 1). They include the major cellular Mg^{2+} influx pathway constituted by: Transient receptor potential melastatin family member 6 and 7 (TRPM6 and TRPM7), and the major Mg export mechanism represented by solute carrier family 41 member A1 (SLC41A1). Many other proteins were described as being directly or indirectly involved in Mg homeostasis such as cyclin and CBS domain divalent metal cation transport mediator 2 (CNNM2) and magnesium transporter 1 (MagT1). The number of publications concerning cellular Mg transport and cellular Mg homeostasis is growing, together with a certain amount of uncertainty, especially about their functions [5-8].

Although the underlying mechanisms are not known, plasma Mg is kept within the range of 0.9-1.2 mmol/L, provided that the influx via absorption from the forestomachs (rumen and reticulum) into the extracellular space is larger than the efflux into the soft tissue and bones for fetal growth during pregnancy, milk production, and intestinal and urinary endogenous secretion [9]. Mobilisation of Mg from bone is unlikely because the ratio between Ca: Mg is 42:1 which would disrupt Ca homeostasis [10]. Therefore, absorption from the forestomach is probably the key factor determining plasma Mg

levels, which can only be kept constant when the daily requirement is adequately balance by ruminal absorption [9]. Accordingly, during the TP ewes become more susceptible to a wide range of metabolic disorders such as pregnancy toxaemia, hypoglycaemia [11], hypocalcaemia and hypomagnesaemia [12].

From previous studies, oral Mg supplementation increased GLUT4 expression in a diabetic rat model and thereby lowered serum glucose levels to the normal range [13]. Moreover, ewes supplemented with Mg during the transition period showed a higher efficiency in energy regulation as observed by a decreased in plasma NEFA concentration [14], similar findings were obtained in periparturient cows [15]. In contrast, hypomagnesaemia induced by feeding a low Mg/high K diet reduced insulin responsiveness and insulin-mediated glucose disposal [16] and stimulated lipolysis in sheep [17].

During lactation, milk production induces high drainage of minerals and energy from the blood into the mammary gland, therefore, one of the main features of the post-partum period is the redistribution of glucose toward the mammary gland [18]. From this context, peripartal immune suppression might be linked to an insufficient energy supply to immune cells, since many studies reported a reduction in immune cells function such as phagocytosis and lymphocyte proliferation during the lactation period [19-21]. A previous study showed a reduction in surface expression of GLUT1, 3 and 4 in bovine monocytes during the post-partum period [22]. Since Mg is involved in glucose metabolism, we hypothesized that Mg supplementation might influence the expression of glucose transporters (GLUT1, 3 and 4) as well as selected Mg transporter genes such as TRPM6 and 7, SLC41A1, MagT1and CNNM2 in leukocytes during the lactation period.

Table 1 Mg Transporters [23]

Name	Cellular location	Tissue expression	Permeability	Mechanism
TRPM7	Plasma membrane	Ubiquitous	Ba > Ni > Mg > Ca	Channel
MagT1	Plasma membrane	Ubiquitous	Mg > Ba > Fe = Cu	Channel
SLC41A1	Plasma membrane	Ubiquitous	Mg > Sr > Fe > Ba > Cu	Exchanger
TRPM6	Apical plasma membrane	Kidney, intestine	Ba > Ni > Mg > Ca	Channel
CNNM2	Basolateral plasma membrane	Kidney	Mg > Sr > Zn > Cd	Homeostatic factor
SLC41A2	Golgi membrane	Ubiquitous	Mg > Ba > Ni > Ca	Exchanger
CNNM3	Plasma membrane	Ubiquitous	Mg > Fe > Cu > Co	Transporter?
MRS2	Mitochondrial membrane	Ubiquitous	Mg > Ni	Channel
CNNM1	?	Brain	Cu > Mg?	?
CNNM4	Basolateral plasma membrane	Intestine	Mg	Exchanger?

2. Materials and Methods

2.1. Ethic Statement

This study was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Permission no.: 33.19-42502-05-18A359). All procedures involving animals were carried out in accordance with German legislation on animal welfare.

2.2. Animals and feeding regimes

Sixteen healthy pregnant German black headed muttons were randomly divided into two groups: control group (n = 8) and Mg group (n = 8). The ewes were housed in the facilities of the Institute of Farm Animal Genetics, Friedrich-Loeffler Institute (FLI) in Mecklenhorst, Germany. The feeding program and dietary ingredients have been described in detail previously [24]. Briefly, ewes received two meals per day, in the morning 3 kg grass silage/animal and at noon 500 g pelleted concentrate/animal (Raiffeisen Schafe S2, Agravis Niedersachsen-Süd GmbH, Hannover, Germany). After parturition (48 h post-partum), the pellets were increased up to 1200 g/animal/day. The feeding plan started one month ante-partum (a.p.) and was continued until one month post-partum (p.p.). The control group received approximately 0.21% Mg of dry matter (DM) daily a.p. and 0.24% Mg of DM daily p.p.. The ration of the Mg group was additionally supplemented with Mg oxide resulting in a calculated daily Mg intake of 0.30% Mg of DM a.p. and 0.38% Mg of DM, respectively. More detailed about the food components and mineral analysis were provided previously [24].

2.3. Blood sampling

One month post-partum blood samples were obtained by puncture of vena jugularis externa and collected in PAXgene Blood RNA tubes (Becton Dickinson GmbH, Heidelberg, Germany). In accordance with manufacturer's instructions, the tubes were inverted gently eight to ten times and stored for 2 h at room temperature, frozen at – 20°C for 24 h and afterwards stored at – 80°C.

2.4. RNA preparation, cDNA synthesis and real-time- polymerase chain reaction (q-PCR)

Total ribonucleic acid from leukocytes was extracted using the PAXgene Blood RNA Kit (Qiagen® Instruments AG, Hombrechtikon, Switzerland) in accordance with the manufacturer's instructions. Total RNA (200 ng/µl) was reverse transcribed to complementary DNA (cDNA) by using random hexamers, oligo-(dT) primers, and TaqMan®-Reverse Transcription Reagents (Applied Biosystems, Deutschland GmbH, Darmstadt, Germany). Real-time PCR was carried out in a ViiA 7 real-time PCR cycler (Thermo Fisher Scientific Inc., Carlsbad, USA) with Biozym Probe qPCR Kit separate ROX (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Gene specific primers and probes for ribosomal protein S19 (RPS19), GLUT1, 3 and 4, TRPM6 and

7, MagT1, CNNM2 and SLC41A1 are presented in Table 2. Amplification of cDNA was carried out in a final volume of 10 µL containing 6 µL mastermix (RPS19, GLUT1, 3 and 4, TRPM6 and 7, MagT1, CNNM2 mastermix contents were: Biozym mastermix: 4.5 µL, Probe: 0.09 µL (150 pmol/µL), Sense primer: 0.3 µL (20 pmol/µL), Anti Sense primer: 0.9 µL (20 pmol/µL), while for SLC41A1 the mastermix contents were: Biozym mastermix: 3.75 µL, Probe: 0.25 µL (150 pmol/µL), Sense primer: 0.5 µL (20 pmol/µL), Anti Sense primer: 1.5 µL (20 pmol/µL) and 4 µL cDNA (diluted 1:2). The temperature protocol consisted of an initial denaturation at 5 °C for 2 min followed by 40 cycles of 95 °C for 5 s and 58 °C for 2 s. The Ct values of the target genes were normalised to RPS19. All reactions were performed in duplicate and relative gene expression was calculated as calibrated normalised relative quantity (CNRQ) by using the $2^{-\Delta\Delta CT}$ method [25]. Negative controls without cDNA and positive controls with ovine liver cDNA templates were included in all reactions.

Table 2 Primers (sense and anti-sense) and probes used to amplify specific genes of glucose and Mg transporters in ovine leukocytes and of reference gene

Gene	Sense (5´-3´)	Anti-sense (3´-5´)	Probe	Amplicon size	Reference sequence code
SLC41A1	TGGTGTTTCTCTACACCATCAG	TCAAGTACGGGATGGAGAAG	[FAM]-ATGTAGAGCAGGATCAGCACCTGGAGCAGA-[TAM]	186	XM_027976221.1
CNNM2	GCTCCAGAATACTACCTCTACC	GCTTCTACTTCTACTTTCCCC	[FAM]-CGAAACAAACCTGTAGACTACTTCGTTCTCAT-[TAM]	83	XM_004020152.4
MagT1	GCTCAATTTGTAGCTGAAACAC	CACACATTATCTTTCGCTTTCC	[FAM]-ATGTGAAGCTGCTACATCTGACATGGATATTG-[TAM]	124	XM_004022211.4
TRPM6	ACAAACCATTCCTTACACTCC	CGTTGTTGTTATTGTACTTCC	FAM]-TTGACCATTGAGAAGTATATGACGGGGGAG-[TAM]	125	XM_012122819.3
TRPM7	ATACAAGAGGGGAGTTACTGG	GGGCCAAAAACCATATCACAG	FAM]-CTGACCCATCTGTGATAAAGGCAGAAGAA-[TAM]	112	NM_001093785.2
GLUT1	CCACAAGCATCTTCGAGAAGGC	TCCACCACAAACAGCGACAC	[FAM]-CACCATCGGCTCCGGCATCGTCAACA-[TAM]	106	XM_027968628.1
GLUT3	TCGCAGGAGAAGCAAGTCACAG	TAGAACACCGCGTTGATCCCAG	[FAM]-CCCAACTACCGGCAGCCCATCATCATCTCCA-[TAM]	122	NM_001009770.1
GLUT4	AGCCATGAGCTATATCTCC	AAGATGAAGAAGCCAAGCAG	[FAM]-TGGCTTCGTGGCCTTCTTTGAAATTGGCCCT-[TAM]	255	XM_027974995.1
RPS19	GGAAAAGGACCAAGATGGGG	CGAACGAGGCAATTTATTAACC	[FAM]-ACAGAGAGATCTGGACAGAATCGCTGGACA-[TAM]	136	XM_004015294.3

2.5 Leukocyte subpopulations count

Blood was obtained by jugular vein puncture into sodium heparin (CAT) vacutainer tubes (BD Vacutainer systems, Roborough, UK) at one month p.p.. Leukocytes were counted microscopically (Nikon microscope ECLIPSE 80i). Fractions of neutrophils, lymphocytes and monocytes among blood leukocytes were determined flow cytometrically. The obtained percentages were multiplied with the total leukocyte counts to obtain total numbers of these cell types among leukocytes, more detailed were provided previously [26].

2.6 Statistical analysis

The data were expressed as mean ± SEM, n representing the number of animals per group. Unpaired t-test (GraphPad Prism 8 Software, San Diego, CA, USA) was used for comparison between the two groups. Correlations between selected parameters (leukocyte subpopulation counts and the expression of glucose and Mg transport associated genes) were analysed by Pearson correlation coefficient. Differences were considered statistically significant when $P < 0.05$ and, $P < 0.1$ is defined as a tendency.

3. Results

3.1 GLUT1, GLUT3 and GLUT4

Mg supplemented ewes, tended to show higher leukocyte expression of GLUT1 mRNA and a numerical increase in GLUT3 mRNA (GLUT1: $P = 0.087$, GLUT3: $P = 0.25$) compared with the control ewes during the lactation period (Figure. 1), GLUT4 could not be detected in both groups. To verify that GLUT4 was present in the leukocytes, conventional PCR and gel electrophoresis were additionally performed with the same primer pair used in qRT-PCR with cDNA of ovine liver samples as positive control. The specific amplification product of GLUT4 could only be detected in liver samples but not in PCRs with leukocyte cDNA.

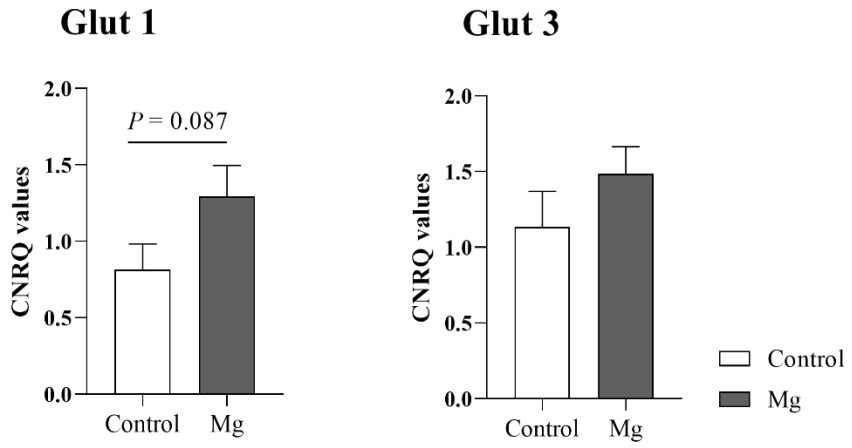

Figure 1. Leukocyte expression of GLUT1 and GLUT3 genes in lactating ewes and the effect of magnesium supplementation. Unpaired t-test was used for the comparison between the two groups. P < 0. 1 is defined as a tendency. Mean ± SEM, (Control group n = 8, Mg group n = 8).

3.2 TRPM6/7, SLC41A1, CNNM2 and MagT1

A numerical increase (P > 0.05) in the expression of TRPM6, TRPM7, SLC41A1, CNNM2 and MagT1 was observed in the Mg group compared to the control group (Figure. 2).

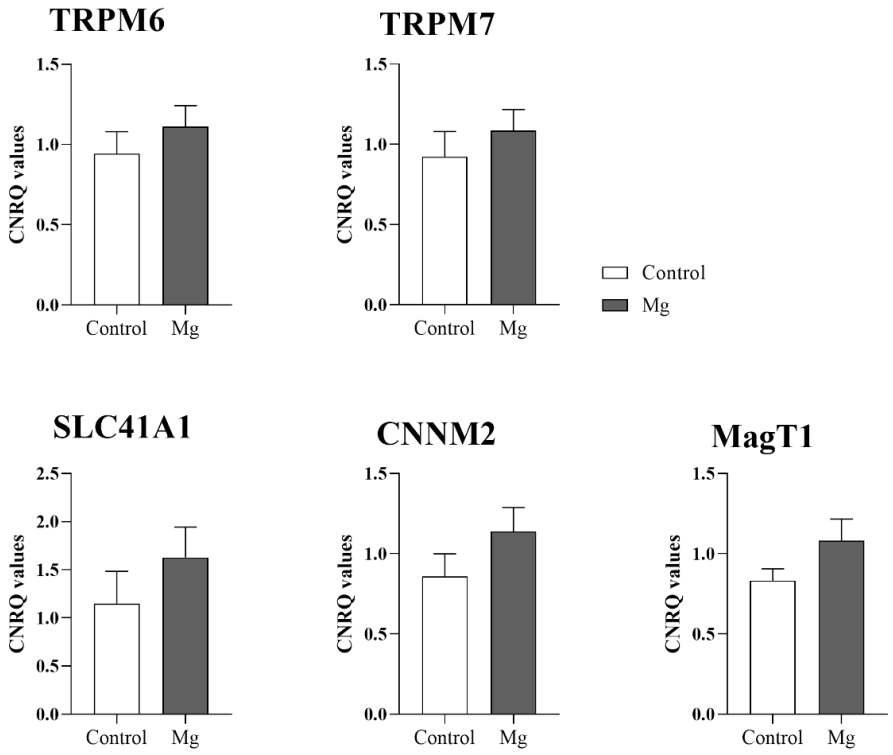

Figure. 2. Leukocyte expression of TRPM6/7, SLC41A1, CNNM2 and MagT1 genes in lactating ewes and the effect of magnesium supplementation. Unpaired t-test was used for

the comparison between the two groups. Mean ± SEM, (Control group n = 8, Mg group n = 8).

3.3 Leukocyte subpopulation numbers and correlations

During the lactation period no significant differences were observed in the numbers of circulating leukocyte subpopulations between the two groups (Figure. 3). The mean values were as following, neutrophils: (Control group: 2.5 ± 0.14 Cells/µL blood (x106), Mg group: 2.6 ± 0.28 Cells/µL blood (x106)), lymphocyte: (Control group: 3.00 ± 0.30 Cells/µL blood (x106), Mg group: 2.9 ± 0.24 Cells/µL blood (x106)), and monocyte: (Control group 47.00 ± 6.0 Cells/µL blood (x104)): , Mg group: 44.00 ± 4.5 Cells/µL blood (x104)).

Moreover, no correlations were observed between certain leukocyte subpopulations and the expression of the investigated genes.

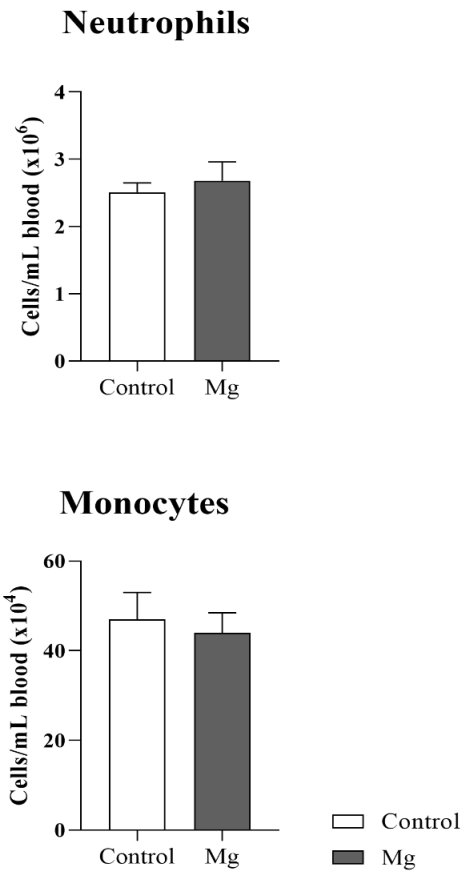

Figure. 3. Numbers of circulation neutrophils, lymphocytes and monocytes in lactating ewes and the effect of magnesium supplementation. Unpaired t-test was used for the comparison between the two groups. Mean ± SEM, (Control group n = 8, Mg group n = 8).

4. Discussion

The results obtained in the present study showed that after two months of dietary Mg supplementation, GLUT1 tended to be upregulated in the Mg ewes compared with the control ewes during the lactation period. No significant differences were observed for GLUT3. Interestingly, GLUT4 could not be detected at the mRNA level in ovine leukocytes. In contrast, previous studies in cows detected GLUT4 in peripheral blood leukocytes [22,27]. We therefore also performed conventional end-point PCR using ovine liver cDNA samples as a positive control, to investigate whether GLUT4 is detectable with our GLUT4 specific primers. This could be confirmed by demonstrating a specific amplification product for GLUT4. These results indicate that GLUT4 expression in ovine leukocytes was below the detection limit in our experimental setup. To the best of our knowledge the effect of Mg supplementation on the expression of glucose transporter genes in leukocytes has not been addressed so far. However, the expression of GLUT4 in skeletal muscles was investigated in rats [13,28], where the authors reported that Mg supplementation increased the expression of GLUT4 in muscle. In the current study, only a numerical increase was observed in the expression of the studied Mg transporter genes during the lactation period in the Mg group.

Studies addressing the influence of Mg supplementation on Mg homeostatic factors/ transporters in leukocyte are scarce. A previous study in humans reported that oral magnesium supplementation significantly increased the leukocyte TRPM6 mRNA relative expression in human leukocytes, whereas there were no changes in TRPM7 and SLC41A1 expression [29]. In another study in periparturient cows the expression of genes associated with Mg transport such as TRPM6/7, SLC41A1/2/3, CNNM2 and MagT1 in kidney, liver, and leukocytes in high vs. low back fat mobilizing cows to assess the potential influence of the intracellular Mg homeostasis in relation to metabolic pathways associated with lipomobilization was investigated [30]. In this study, higher expression of TRPM7 (liver, kidney and leukocytes) was reported in low- mobilizing cows which indicated a better cellular supply of various tissues with Mg in these animals, since TRPM7 represents one of the main entry mechanism for Mg into the cells. Moreover, higher expression of MagT1 was reported in the kidneys of low- mobilizing cows, which could support a more efficient reabsorption of Mg by renal epithelial cells and may contribute to a better whole body Mg status [30]. TRPM6 was reported to be upregulated in response to Mg deficiency, therefore, the mentioned authors speculated that, higher expression of TRPM6 in the liver of high – mobilizing cows might be a further indicator for an insufficient supply in these animals.

Given the central role of Mg in energy metabolism and that this cation is essential for the efficacy of insulin and glucose metabolism, a better Mg status may counteract excessive lipomoblization during peripartal period.

To obtain a more complete picture of the dynamic changes in gene expression, in future studies it will be interesting to monitor expression of glucose transporters and genes involved in Mg homeostasis throughout the whole transition period starting from one month a.p. to one month p.p., furthermore not only expression in leukocytes but also in tissues related directly to Mg homeostasis such as rumen and kidney should be analyzed. Additionally, it would be interesting as well to investigate these genes in hypomagnesemic ewes vs. normomagnesemic ewes.

5. Conclusions

In the present study, the tendency to show higher leukocytes expression of GLUT1 as well as the numerical increases in mRNA expression of GLUT3, TRPM6/7, SLC41A1, CNNM2 and MagT1 in ewes offered more Mg, suggest a suitability of additional Mg in the transition period to promote glucose metabolism and Mg status in lactating ewes. Future research addressing the relationship between different concentrations of Mg intake and the expression of these genes in leukocytes, rumen, liver and kidney is needed.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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7 General discussion

Effects of magnesium supplementation during the transition period on metabolic profile, immune cell functions and vaccination response in ewes

7.1 Main findings and discussion

Transition from late pregnancy to early lactation is characterized by high energy demand, nutrient deficiency, mineral imbalance and hormonal fluctuations which could interfere negatively with body homeostasis and particularly with the immune system. Thus, the main features of this period are potential metabolic disorders and immune dysregulation. Since Mg is required for a huge number of metabolic reactions in addition to its role as an immune modulator, we hypothesized that dietary Mg supplementation modulates mineral homeostasis and improves glucose metabolism as well as insulin function in ewes during the TP. Furthermore, .it alters distinct immunological parameters such as blood leukocyte composition, neutrophil phagocytosis, lymphocyte proliferative capacity *in vitro*, and the response to vaccination.

The preliminary results have shown that ewes during the transition period had a lower fraction of phagocytic-positive neutrophils compared with the non-pregnant ewes, moreover the percentages of monocyte subpopulations (classical, intermediate and nonclassical monocytes) in the peripheral blood showed a different pattern during the TP in comparison with non-pregnant ones (Appendix: A-B). Regarding the serum biochemical parameters, higher serum levels of BHB as well as lower levels of total protein levels were observed in the ewes during the TP in comparison with non-pregnant ewes (Appendix: C-D). These findings helped us to set-up the main experiment.

In the first part of this PhD project, the influence of Mg supplementation on mineral levels and intermediary metabolism parameters as well as protein parameters were investigated. As pointed out in the first manuscript, serum levels of Ca, P_i, glucose and insulin fluctuated more prominently throughout the TP in the control group compared with Mg group.

Moreover, Mg levels stayed stable throughout the TP in both groups and we observed no significant differences in Mg serum levels between the control and the Mg group, which is in line with previous studies in periparturient cows supplemented with Mg (Leno et al., 2017; Tebbe et al., 2018). This may have been due to the fact that differences in bioavailability of Mg would likely be more reflected in the urine than in the plasma/serum (Jesse et al., 1981; Van Ravenswaay et al., 1989). Serum Mg concentrations do not always

increase in response to feeding mineral sources with apparently higher Mg bioavailability (Xin et al., 1989); however, strong linear correlations between apparently absorbed Mg and urinary Mg as well as serum Mg have been reported in sheep (Chicco et al., 1972) indicating that these measures provide insight into the Mg absorbed from the diet. Secondly, in this experiment we only measured the total Mg levels, however, the ionized Mg is the physiologically-active form (Altura and Altura, 2016) Therefore, measuring the ionized Mg might provide a more detailed on the accurate status of the Mg and its metabolic activity.

As discussed in the first manuscript, at d 1 p.p., a sharp decrease in P_i serum levels was observed only in the control group, which was similar to a previous study in cows supplemented with Mg during the TP (Leno et al., 2017). The mentioned auother observed lower serum levels of P_i only in unsuplemented cows. This species similarity could be explained by the following two aspects. Firstly, it could be due to high cortisol level (parturition stress) which is correlated negatively with P_i level (Horst and Jorgensen, 1982). From a previous study, Mg was reported to decrease cortisol levels (Dmitrašinović et al., 2016), however, this explanation is diffcult to be considered in this experiment since there was no typical increase in cortisol levels around parturition in our animals. Secondly, it is well documented that Mg is involved in PTH and 1, $25(OH)_2$ D_3 function, which are essential for Ca and P_i homeostasis (Ataollahi, 2018; Rude et al., 2009), therefore Mg supplementation might be reflected positivily in the ewes offered more Mg.

In the current study, serum glucose levels were more stable throughout TP in the Mg group in comparison with the control group. This observation might confirm the role of Mg in the glucose metabolism as reported previously in humans and hypomagnesemic sheep (Matsunobu et al., 1990; Meludu and Adeniyi, 2001). In ruminants, the major part of glucose must be synthesized by gluconeogenesis in the liver using propionate as main substrate (Aschenbach et al., 2010); where Mg is required as a coenzyme for most of the enzymes involved the gluconeogenesis such as pyruvate carboxylase (Utter and Keech, 1963), phosphoenolpyruvate carboxykinase (Foster et al., 1967). In addition, Mg participates in the transport of glucose in insulin-dependent transport mechanisms across the cell membrane (Rodríguez-Morán and Guerrero-Romero, 2003), which increases the tissue's glucose uptake and sequentially decreasing the insulin and glucose levels in the circulation (Manuscript 1).

Furthermore, we observed a negative correlation between the glucose and BHB levels as well as a positive correlation between glucose and insulin levels only in the control ewes

before lambing, our findings are in accordance with what was reported previously by Duehlmeier et al, (2011) in GMB ewes, interestingly these correlations were missing in the Mg group. The lack of such correlations in the Mg group might indicate a modulation of glucose metabolism. Therefore, it could be speculated that GMB ewes offered more Mg were able to maintain to a considerable extent stable glucose levels throughout the TP compared to the control group, which might decrease their susceptibility to pregnancy toxemia (Manuscript 1).

It has been well documented that parturition is associated with a remarkable alteration in nutrient and metabolic demand as well as hormonal changes (Goff and Horst, 1997; Meglia et al., 2001), which can be associated with impaired immune function as we observed in the preliminary experiment as well as reported in previous studies (Preisler et al., 2000b; Tang et al., 2015).

Therefore, in the second part of this PhD project, we hypothesized that Mg supplementation during the TP modulates distinct immunological parameters such as blood leukocyte composition, neutrophil phagocytosis, lymphocyte proliferative capacity, and the response to vaccination.

In the present study, ewes supplemented with Mg showed both a significant higher percentage of phagocytosis-positive neutrophils around parturition, as well as a higher phagocytic capacity per cell compared with the control ewes (Manuscript 2). Comparable studies are scarce. Our findings are supported partly by *in vitro* data obtained from a monocyte-like human lymphoma U937 cell line where extracellular Mg levels correlated positively with an enhanced phagocytosis rate (Diler et al., 2015). The enhancement in the phagocytic potential of neutrophils could be due to the enhancement in glucose viability and cellular uptake in the Mg group (Manuscript 1).

We extended the analysis of Mg supplementation effects on immune functions to the proliferative capacity of lymphocytes and this function appeared to be rather stable throughout the TP when considering the proliferation of all lymphocytes with no apparent influence of Mg supplementation (Manuscript 2).

As discussed earlier in the second manuscript, the analysis of proliferating T lymphocyte subpopulations ex vivo revealed some interesting details. In comparison with the control group, the proliferative response of CD4+ T cells in Mg-supplemented ewes was significantly higher throughout the whole TP. In contrast, the effects of Mg supplementation were less prominent on the proliferative capacity of CD8+ T cells,

pointing towards a specific role of Mg for T-cell subpopulations. The enhanced CD4+ T cell proliferation could be due to the improvement in glucose availability and cellular uptake (Manuscript 1). Moreover, the role of Mg in lymphocyte intracellular signalling pathway activation has been previously reported in human patients diagnosed with a mutation in an Mg transporter gene MagT1 (novel X-linked human immunodeficiency). This condition is characterized by hypomagnesaemia, CD4+ lymphopenia and defective T-lymphocyte activation (Li et al., 2011). Where Mg plays a role in the activation of T-lymphocytes is still under discussion. Mg may be involved in T cell receptor activation (Li et al., 2011), the glycolysis process (Sahni et al., 2010), or in the activation-induced Ca influx which partially depends on Mg (Li et al., 2011).

Regarding blood leukocyte cell numbers, ovine neutrophil and lymphocyte numbers during TP showed the same pattern as in cows whereas monocytes and their subsets behaved strikingly different in periparturient ewes. Several factors affecting leukocyte numbers around parturition were reported in cows for instance hormonal changes (e.g. cortisol level) (Menge and Dean-Nystrom, 2008), as well as bone marrow activity and chemokine generation in tissues which could be linked to the bovine monocytosis around parturiton. In ewes, as presented in the second manuscript, monocytes and their subsets definitely peaked later at one month post-partum. Although not studied in further detail, it could be speculated that the mentioned factors guiding the redistribution of monocytes and their subsets might not been active in periparturient ewes and that other factors are responsible for the divergent redistribution of ovine myeloid cells (neutrophils, monocytes) during TP. Further studies are needed to investigate this phenome in ewes. Notably, the supplementation with Mg did not alter any of the cell-type-specific redistribution pattern (Manuscript 2).

We went one step further and questioned whether Mg supplementation also affects complex immune responses depending on the complex interplay of soluble mediators and different cell types *in vivo*. This was addressed by analysing the immediate cellular response within 24 h following vaccination and the humoral immune response against *Mycobacterium avium subsp. paratuberculosis* (MAP) at days 7 and 21 post-vaccination (Manuscript 2). In the present study, the vaccination resulted in a significant increase in MAP-specific antibody levels at d 7 and d 21 post-vaccination. This rapid increase in IgG antibodies against MAP might have been mediated by a booster response of ewes previously infected with MAP (Harris and Barletta, 2001). Since there were no significant differences in the antibody response between control and Mg supplemented ewes, this

argues against an influence of Mg supplementation on the cascades leading to an activation of existing T and B memory cells (Manuscript 2).

Furthermore, effects of vaccination could already be observed within 24 h after vaccination. Blood total leukocyte, neutrophil and ncM numbers significantly increased whereas total numbers of blood monocyte and cM dropped post vaccination in both groups. Interestingly, significant increase in intM numbers was observed only in the Mg group, as well as significant decrease in lymphocytes which might indicate a selective influence of Mg supplementation (Manuscript 2).

The very early changes in the numbers of circulating leukocyte populations following a vaccination has just been reported in a recent study in beef steers (Hughes et al., 2017), where an increase in total leukocytes, neutrophils and monocytes, together with a decrease in lymphocyte numbers was reported within 24 hours following a multivalent combination respiratory vaccine of animals treated with dexamethasone (acute stress), while the number of the circulating blood cells remained stable post vaccination in the control animals. Moreover, in a much more recent study (Hudson et al., 2020) the previous mentioned pattern of the changes in circulating leukocytes composition has been observed in both stressed and control vaccinated beef steers.

As discussed in the second manuscript, the observed changes in blood leukocyte subpopulation numbers are most likely due to distal effects of the spectrum of vaccination-induced mediators. Thus, differences between the control and the Mg-supplemented group might point towards an influence of dietary Mg on the regulated release of such factors from vaccine/adjuvant-triggered cells, e.g. dendritic cells as has been reported previously (Libako et al., 2015; Schempp et al., 2000). To the best of our knowledge, the influence of Mg on the early innate immune response following vaccination has not been addressed so far.

In the third part of this PhD project, we investigated whether the gene expression of the glucose transporters (GLUT1, 3 and 4) as well as selective Mg transporters such as SLC41A1, CNNM2, TRPM6, TRPM7, and MagT1 in leukocytes were modulated after two months of dietary Mg supplementation.

As discussed in the third manuscript, GLUT1 tended to be upregulated in the Mg group, while the leukocyte expression of GLUT3, SLC41A1, CNNM2, TRPM6, TRPM7, and MagT1 showed only numerical increase compared to the control group, however, no signals were detected for GLUT4 in leukocytes in both groups. Studies addressed the effect of Mg supplementation in the expression of glucose and Mg transporter genes are scarce.

Two studies in mice reported that Mg supplementation improved the expression of GLUT4 in muscle (Morakinyo et al., 2018; Solaimani et al., 2014). Regarding the Mg transporters, one study in humans revealed that oral Mg supplementation significantly increased the leukocyte TRPM6 mRNA relative expression, whereas there were no changes in TRPM7 and SLC41A1 (Rodríguez-Ramírez et al., 2017). In the present study we succeeded in establishing qRT-PCR protocols to investigate the mentioned genes in ovine leukocytes. To get more information regarding the influence of Mg supplementation on the expression of Mg transporter genes, it is worthwhile to investigate these parameters throughout the whole transition period starting from one month a.p. to one month p.p., especially in tissues related directly to Mg homeostasis such as rumen and kidney.

7.2 Physiological portability

In view of the practical relevance of the findings of this PhD project, the obtained results supported the influence of Mg supplementation on glucose metabolism in periparturient GMB ewes, which might decrease their susceptibility to pregnancy toxaemia. Additionally an enhancement in the P_i levels around parturition was observed as well in the supplemented animals. Moreover, achieved findings shed new light on the role of Mg as an immune modulator since enhancement in neutrophil phagocytosis and T-lymphocyte subsets (CD4+) proliferation *in vitro* was reported as well as a modulation in the immediate vaccination response *in vivo*.

Metabolic changes and immunological dysregulation during the TP are highly comparable in ewes and cows, and with special regard to dairy cows representing a huge proportion of livestock. Therefore, the modulation in glucose, insulin, P_i serum levels as well as the immunological parameters might enhance the ability of periparturient cow to cope with such a critical period, particularly around parturition since the pregnant animal become highly susceptible to Mg deficiency.

In humans, global multiple epidemiologic data over the past 100 years indicate a decline of daily Mg intakes. Different factors for higher daily Mg intake must be considered. For instance, high dietary intake of calcium, phosphate, vitamin D, or even high protein diets will result in a greater daily Mg requirement in order to maintain a positive Mg balance. In addition, environmental stress, high fat or high carbohydrate diets, refined grains and processed food will increase the need for higher than normal daily intake of Mg.

A close association between hypomagnesemia and Type 2 diabetes has been established; and the role of Mg supplementation in the management of glucose homeostasis has been

often reported. Similarly, from clinical studies was concluded that hypomagnesemia is associated with insulin resistance in both diabetic and non-diabetic healthy individuals. Therefore, an adequate daily intake of Mg is a relevant issue for human health as well.

7.3 Evaluation of PhD project and further studies

In the first part of the PhD project it was shown that ewes offered more Mg were able to maintain stable serum levels of P_i and glucose throughout the TP compared with the control ewes. With regard to the total Mg levels no significant differences were observed between the two groups.

The classical implication of high K intake for the inhibition of Mg absorption is well known in ruminants (Fontenot et al., 1960; Schonewille et al., 1999). In the present study the K daily intake (4.27 % of DM ≈ 35.6 g a.p, 42.6 g p.p.) was higher than NRC (2007) recommendations, however, the animals in both groups were normomagnesaemic. Our observations were similar to what was reported previously in cows fed silage with high K contents within a range between 30-45 g/kg DM (Schonewille et al., 1997). Firstly, this might have been due to the fact that high levels of Mg in the whole ration might have interfered with the inhibitory action of K on Mg ruminal absorption. Secondly, the typical anion moiety of K salts occurring in grass silages (as fertilizer) might not allow for the inhibitory effect of K on Mg absorption to be expressed (Schonewille et al., 1997).

In the second part, as it was shown that dietary Mg supplementation does not interfere to a large extent with the circulation behavior of immune cells. In fact, it selectively favours the functional capacity of neutrophils and T-lymphocyte subsets during the TP. The impact of dietary Mg on the composition of circulating immune cells after vaccination against MAP suggests that Mg modulates early vaccine-induced innate immune mechanisms.

For further studies, it would be of interest to measure the serum levels of ionized Mg besides the total Mg. Moreover, to prove the influence of Mg supplementation on P_i serum levels around parturition it would be interesting to determine the serum levels of PTH and $1,25(OH)_2D_3$.

Regarding the high energy demand during the transition period and the role of Mg, analyzing none esterified fatty acids levels in serum would provide more information about the manipulation in the glucose metabolism especially around parturition. Furthermore, investigating the expression of GLUT4 in the muscle tissue would provide more evidence regarding the influence of Mg on insulin function. Additionly, for further studies it is

important to take into consideration the expression of Mg transporter genes in rumen and kidney which might give more information about the Mg handling in the body. Lastely, the modulatory effects of Mg on early vaccine/pathogen-induced innate immune responses needs to be studied in more depth at intracellular signalling pathways level.

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Affidavit

I herewith declare that I autonomously carried out the PhD thesis entitled “Effects of magnesium supplementation during the transition period on metabolic profile, immune cell functions and vaccination response in ewes”.

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at Institute for Physiology and Cell Biology, University of Veterinary Medicine Hannover

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

_____

[date], signature

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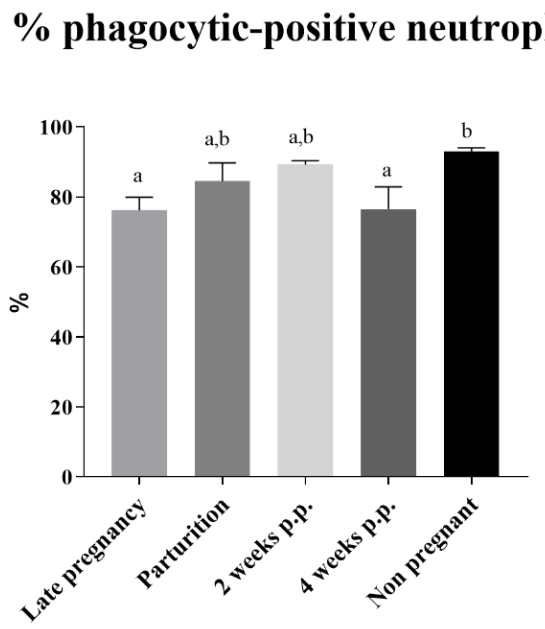
Appendices

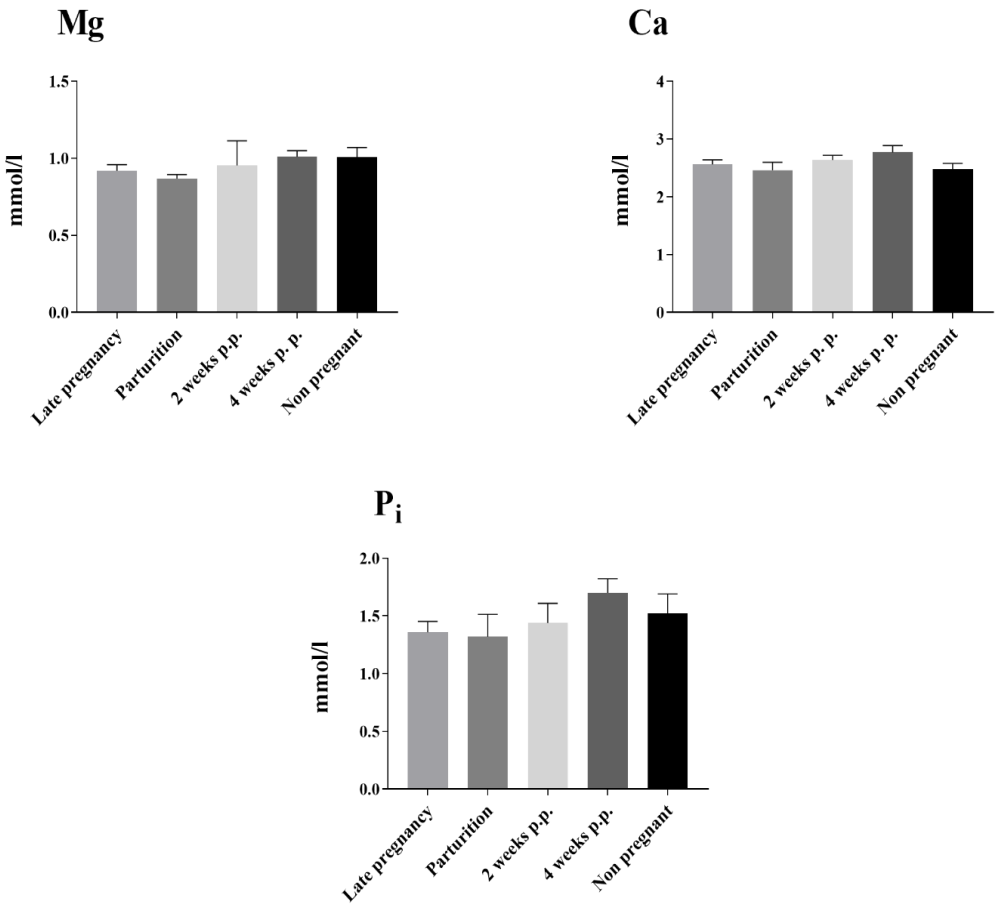
Preliminary work results



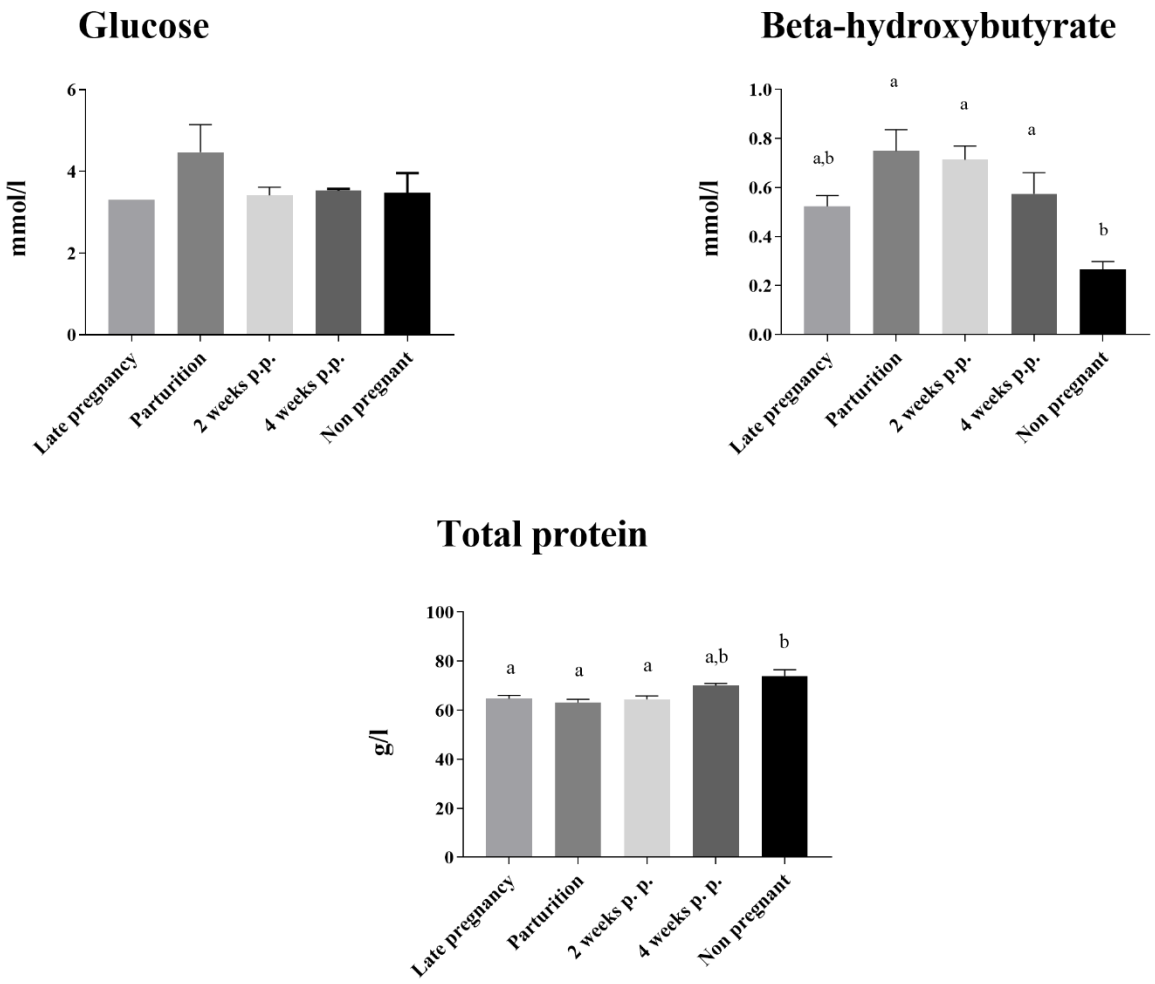
Appendix A. Percentage of phagocytic- positive neutrophil *in vitro* in periparturient (during transition period) and non-pregnant German black headed mutton ewes. One way-ANOVA. periparturient ewes n = 5, non-pregnant ewes n = 5, mean ± SEM. Significant time-dependent differences ($P < 0.05$) are indicated by different letters. p.p: post-partum.

Appendix B. Monocyte subset percentages of non- pregnant and periparturient German black headed mutton ewes. Mean values within the same column bearing different letters are significantly different at $P < 0.05$ (Unpaired t-test).

Groups	cM%	intM%	ncM%
Non-pregnant ewes (n=5)	70 ± 16	13 ± 5[a]	17 ± 12
Periparturient ewes (n=5)	53 ± 17	27 ± 11[b]	20 ± 13



Appendix C. Serum levels of magnesium (Mg), calcium (Ca) and phosphate (P_i) in periparturient (during transition period) and non-pregnant German black headed mutton ewes. One way-ANOVA. periparturient ewes n = 5, non pregnant ewes n = 5, mean ± SEM. p.p.: post-partum



Appendix D. Serum levels of glucose, beta-hydroxybutyrate and total protein in periparturient (during transition period) and non-pregnant German black headed mutton ewes. One way-ANOVA. periparturient ewes n = 5, non-pregnant ewes n = 5, mean ± SEM. Significant time-dependent differences, $P < 0.001$) are indicated by different letters. p.p: post-partum.