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Post calving metabolic status dependent changes on expression of extracellular vesicle coupled microRNAs in bovine follicular fluid and blood serum

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Dedicated to my beloved mother and my lovely sons

Post calving metabolic status dependent changes on expression of extracellular vesicles coupled microRNAs in bovine follicular fluid and blood serum

Most high yielding dairy cows enter a state of negative energy balance (NEB) during early lactation. This phenomenon is associated with disturbed metabolic status of the cows reflected in the blood and follicular fluid (FF) microenvironment leading to ovarian dysfunction. Extracellular vesicles (EVs) are evolutionarily conserved communicasomes with cargo molecules found in most biological fluids including follicular fluid and they transport different bioactive molecules such as miRNAs, proteins, and lipids from donor to recipient target cells. This dissertation is aimed to investigate the association between post-calving metabolic status and the expression of EV-coupled miRNAs in FF and blood serum of Holstein-Friesian cows. Moreover, the effect of lactation physiology on the expression of EV-coupled miRNAs in FF and blood serum was conducted by comparing lactating cows and heifers. For this, FF samples were collected from large follicles using ovum pick-up procedure from cows (n=30) at weeks 5-10 of post-calving. FF samples collected from heifers (n=8) were used as a control. Parallel to FF collection, blood samples were collected from all experimental animals. Screening of the energy balance status of cows was performed using blood metabolite concentration, body weight, and overall energy based on dry matter intake prior to sample pooling. Following this, EVs were isolated from pooled FF and serum samples using ultracentrifugation and miRNAs expression analysis was performed using Illumina based Next-generation sequencing. Results of energy balance analysis revealed that the majority of the cows (66%) were transient (TCs), whereas the remaining cows exhibited either always negative (ANCs) (17%) or always positive (APCs) (17%). Sequencing results revealed that, a total of 356 and 179 known miRNAs across all samples were detected in FF and serum samples, respectively. Hierarchical clustering and pairwise differential expression analysis of EV-coupled miRNAs in FF and serum revealed major downregulation of miRNAs in metabolically stressed cows. Furthermore, specific EV-coupled miRNAs (bta-miRNA-122, bta-miRNAs-150, bta-miRNA-9-5p, and bta-miRNA-196) were commonly detected with similar pattern of expression in ANCs vs heifers in serum and FF. Moreover, comparison of APCs vs heifers revealed that, lactation has induced the expression of EV-coupled miRNAs in FF but not in the blood serum. In conclusion, cows with divergent metabolic status showed distinct pattern of expression of EV-coupled miRNAs, which can be excellent input for further studies aiming at development of noninvasive molecular indicator of metabolic stress in dairy cows.

Veränderungen der Expression extrazellulärer Vesikel-gekoppelter microRNAs in Rinderfollikelflüssigkeit und Blutserum in Abhängigkeit vom Stoffwechselstatus nach dem kalben

Die meisten Hochleistungsmilchkühe geraten während der frühen Laktation in einen Zustand der negativen Energiebilanz (NEB). Dieses Phänomen ist mit einem gestörten Stoffwechselstatus der Kühe verbunden, der sich in der Mikroumgebung von Blut und Follikelflüssigkeit (FF) widerspiegelt und zu Funktionsstörungen der Eierstöcke führt. Extrazelluläre Vesikel (EVs) sind evolutionär konservierte Signalträger die mit verschiedenen Molekülen beladen sind. Sie kommen in den meisten biologischen Flüssigkeiten, einschließlich Follikelflüssigkeit und transportieren verschiedene bioaktive Moleküle wie miRNAs, Proteine und Lipide von der Spender- zur Empfängerzielzelle. In dieser Dissertation sollte der Zusammenhang zwischen dem Stoffwechselstatus nach dem Abkalben und der Expression von EV-gekoppelten miRNAs in FF und Blutserum von Holstein-Friesian-Kühen untersucht werden. Darüber hinaus wurde die Auswirkung der Laktationsphysiologie auf die Expression von EV-gekoppelten miRNAs in FF und Blutserum durch Vergleich von laktierenden Kühen mit Färsen analysiert. Zu diesem Zweck wurden FF-Proben von großen Follikeln in den Wochen 5 bis 10 nach dem Abkalben, unter Verwendung der Ovum pick up Methode, von Kühen (n = 30) entnommen. FF-Proben von Färsen (n = 8) wurden als Kontrolle verwendet. Parallel zur FF-Entnahme wurden von allen Versuchstieren Blutproben entnommen. Das Screening des Energiebilanzstatus von Kühen erfolgte durch die Analyse der Blutmetabolitenkonzentration, des Körpergewichts und der Gesamtenergie basierend auf der Trockenmasseaufnahme vor dem Sammeln der Proben. Anschließend wurden EVs aus gepoolten FF- und Serumproben mittels Ultrazentrifugation isoliert, und die Expressionsanalyse der miRNAs wurde unter Verwendung von Illumina-basierter Next-Generation-Sequenzierung durchgeführt. Die Ergebnisse der Energiebilanzanalyse ergaben, dass die Mehrheit der Kühe (66%) Transient (TCs) waren, während die übrigen Kühe entweder immer negativ (ANCs) (17%) oder immer positiv (APCs) (17%) waren. Die Sequenzierungsergebnisse zeigten, dass insgesamt 356 und 179 bekannte miRNAs in allen FF- bzw. Serumproben nachgewiesen wurden. Das hierarchische Cluster- und die paarweise differentiellen Expressionsanalysen von EV-gekoppelten miRNAs in FF und Serum ergaben eine starke Herunterregulierung von miRNAs in metabolisch gestressten Kühen. Darüber hinaus wurden spezifische EV-gekoppelte miRNAs (bta-miRNA-122, bta-miRNAs-150, bta-miRNA-9-5p und bta-miRNA-196) häufig mit ähnlichen Expressionsmustern in ANCs im Vergleich zu Färsen in Serum und FF nachgewiesen. Weiterhin ergab der Vergleich von

APCs mit Färsen, dass die Laktation die Expression von EV-gekoppelten miRNAs in FF induziert hat, nicht jedoch im Blutserum. Zusammenfassend lässt sich festhalten, dass Kühe mit unterschiedlichem Stoffwechselstatus ein deutliches Expressionsmuster für EV-gekoppelte miRNAs aufweisen. Dies ist eine wichtige Erkenntnis für weitere Studien, die auf die Entwicklung eines nicht-invasiven molekularen Indikators für metabolischen Stress bei Milchkühen abzielen.

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List of abbreviations

AI	Artificial insemination
Alix	ALG-2 interacting protein X
ANCs	Always negative cows
APCs	Always positive cows
BHO	Beta–hydroxybutyrate
CD63	CD63 molecule
cDNA	Complementary DNA
COC	Cumulus-oocyte-complex
DAPI	6-Diamidin-2’-phenylindoldihydrochlorid
DE	Differentially expressed
EVs	Extracellular vesicles
FF	Follicular fluid
IVF	In vitro fertilization
kDa	Kilo Dalton
LH	Luteinizing hormone
miRNAs	MicroRNAs
mRNA	Messenger RNA
ncRNA	Noncoding RNA
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
OPU	Ovum Pick Up
PBS	Phosphate-buffered saline
PBS-CMF	Ca2+/Mg2+ free 1x phosphate buffer saline
RIPA	Radioimmuno precipitation assay buffer

TCs	Transient cows
TMM	trimmed mean of M-values

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Chapter 1: General overview

1.1 Introduction

1.1.1 Mammalian Reproduction

Every living organism is able to leave its copy on the earth by the dogma of reproduction. Reproduction is the ‘sine qua non’ for the propagation of species and the continuation of life (Roy & Matzuk 2006). In mammals, reproduction starts from the process of gametogenesis, which is the formation of the oocyte for females and the formation of sperm for the males. The process of reproduction is evolutionary conserved across different mammalian species and many other vertebrates, including amphibians, reptiles, birds and fish (Matzuk & Lamb 2002). Gametes (oocyte and sperm) cells are uniquely specialized to transmit genomic information to succeeding generations upon fertilization and give rise to the embryo (Matzuk *et al.* 2002). Production of future generations with genetically and functionally competent gametes is a prerequisite for normal fertilization and early embryo development. It had well established that oocytes and spermatozoa initiate from the same cell types. The germ cell of an embryo of both gametes serves to produce a haploid genome ready for fertilization and the creation of a new diploid offspring (Spiller *et al.* 2017).

Studies on male reproduction in the last century have focused on testicular cell culture and the first germ cell culture systems directed to studying gonadal embryogenesis and male germ cell differentiation (Staub 2001). However, little has been known on the importance of cell to cell communication in spermatogenesis by clinical cases of male infertility, transgenic mouse models, and xenogenic germ cell transplantation experiments (Matzuk *et al.* 2002). In female reproduction, the ovarian follicle has been confirmed as the fundamental unit of the ovary consisting of both the oocyte, that undergo ovulation, fertilization and form an embryo, and the surrounding somatic cells, which produce signals and hormones to accomplish the developmental competency. At birth, the oocytes interacted with granulosa cells during the

formation of the primordial follicles and after puberty, the oocytes grow as the surrounding granulosa cells proliferate and differentiate (Amleh and Dean 2002).

Regardless of the magnificent study on the improvement of reproductive efficiency across the globe, the number of competent oocytes that riches the blastocyst stage is very low. In bovine in vitro development, 90% of immature oocytes have been documented to reach metaphase II and extrude the first polar body, followed by 80% of them undergo fertilization, cleavage and reach until two-cell stage, however only 30-40% reaches the blastocyst stage (Lonergan *et al.* 2001). In human, approximately 15% of couples in the United States have been reported as unable to conceive within one year of unprotected intercourse (Matzuk & Lamb 2002). One of the main factors associated with this declining fertility is the lack of oocyte and embryo competency. The term developmental competence of an oocyte in cattle is defined as the ability to resume meiosis, to cleave upon fertilization to develop into a blastocyst, to induce pregnancy and to generate a healthy offspring (Sirard *et al.* 2006). Similarly, the best measure of blastocyst quality is the ability to establish a pregnancy and produce a calf (Lonergan *et al.* 2001). With this regard, the malefactor has been reported to be responsible for only 20% of overall infertility in humans (Vg & Ramgir 2015).

1.1.2 Causes of mammalian infertility

Fertility is a complex process that is affected by species, gender, individuality, genetics, environment, and possibly others. Therefore, the overall reproductive success is when one measured on the production of healthy offspring capable of propagating the species (Foote 2003). According to the International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of 2009, the term “Infertility” is clinically defined as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse in human (Zegers-Hochschild *et al.* 2009). For many couples, the inability

to give birth to a child is a tragedy and the conflux of personal, interpersonal, social, and religious expectations brings a sense of failure, loss, and exclusion to those who are infertile (Rutstein & Shah 2004). Few genetic causes of infertility had identified in humans, mice, and cattle, while environmental factors play a significant role. Defects in sexual differentiation pathways have been reported to cause infertility in mice and humans of both sexes (Matzuk and Lamb 2002). Although approximately 13% of American women will receive infertility services during their lifetime4, only 1–2% will undergo treatment with ARTs (Katz *et al.* 2002). Progress in the field of reproduction study has been realized to understand the genetic regulation of fertility, with the production of over 400 mutant mouse models with a reproductive phenotype and with the promise of regenerative gonadal stem cells (Matzuk and Lamb 2008). However, in large animals like cattle, most of the genetic components' fertility problems have not discovered still.

In cattle, the causes of pre-implantation embryo loss for more than 50% of fertilized embryos have been documented as multifactorial and largely not described yet (Hansen *et al.* 2004). In general, the reason for declining fertility in lactating cows could be due to; anovulatory and behavioral anestrus (failure to cycle and display estrus), suboptimal and irregular estrous cyclicity, abnormal preimplantation embryo development, and uterine/placental incompetency (Lucy 2007). Therefore, in dairy cows, several hypotheses have been speculated to explain the reduction of fertility issues such as genetics, physiology, nutrition and management, and these factors have been investigated at the animal, organ and cellular level at critical time points of the productive life of dairy cows (Walsh *et al.* 2011). As future strategies, studies suggested to give high priority and to put efforts on holistic approaches by minimizing, negative energy balance and resolve any infection of the postpartum uterus, expression and detection of oestrus followed by insemination with high quality semen, ovulation and fertilization of a high quality oocyte, an increase in progesterone secretion from the corpus luteum, uterine

endometrium must produce an early and appropriate environment to stimulate embryo implantation, and large embryo producing adequate quantities of interferon tau that alters uterine prostaglandin secretion and signals maternal recognition of pregnancy (Walsh *et al.* 2011).

1.1.3 Trends in dairy cow fertility

The most applause technology in the dairy industry is the invention of assisted reproduction techniques that revolutionized production in the last couple of decades. It is mainly after the invention of artificial insemination as the first reproductive technology applied to cattle in Russia and Denmark during the early 1900s. This is due to its potential to accelerate the rate of genetic gain in livestock populations by the widespread use of sires with elite genetic merit and has grown to internationally giving service up to 130 million cattle per year (Moore & Hasler 2017). This revolution was further a few years later accompanied by semen sorting for sex determination, embryo transfer, and cryopreservation of gametes facilitated the selection of animals on the dairy production. Following this, multiple ovulation and embryo transfer also become the most cost-effective way to exploit female genetics in cattle and small ruminants (Galli, 2017). Improvement in genetic selection associated with improvement in management and nutrition has steadily increased the milk production per cow per year (Lucy 2001). In this regard, a 50 years study showed that milk production has increased from 2000 kg in the 1950s to 12000 kg per lactation in 2001, however, conception rate continuously reduced from ≈70% in 1951 to ≈30% in 2001 (Butler 2003). Similarly, the pregnancy rate has been declined from 50–60% in the 1970s to 35–45 % (Royal et al. 2000). Currently, declining fertility is believed to be a leading cause of poor productivity and limits the target being that every cow produces one calf a year (Ioannidis & Donadeu 2016). One of the reasons with the higher share has documented to be postpartum negative energy balance during early lactation.

1.1.4 Impact of negative energy balance on dairy cows' fertility

In modern dairy farms, most if not all cows go through some period of negative energy balance (NEB) during early lactation (Royal *et al.* 2000, Butler 2003, McGuire *et al.* 2004, van Knegsel *et al.* 2007). The term "Negative Energy Balance" in high producing dairy cows is defined as a condition during their first one-third of lactation in which the energy intake and requirements for a lactation and maintenance is higher than the total energy intake and those cows use the additional energy from body reserves to meet their need (Bauman & Currie 1980). In the high genetic merit dairy cow, the post-calving negative energy balance is a physiological phenomenon that results in severe disorders in both the metabolism and reproduction and causes great economic losses in modern dairy practice (Huszenicza *et al.* 2004). Different energy supply and requirements during the lactation cycle of 340 days in cows are summarized in Fig 1.1 (Strucken et al. 2015).

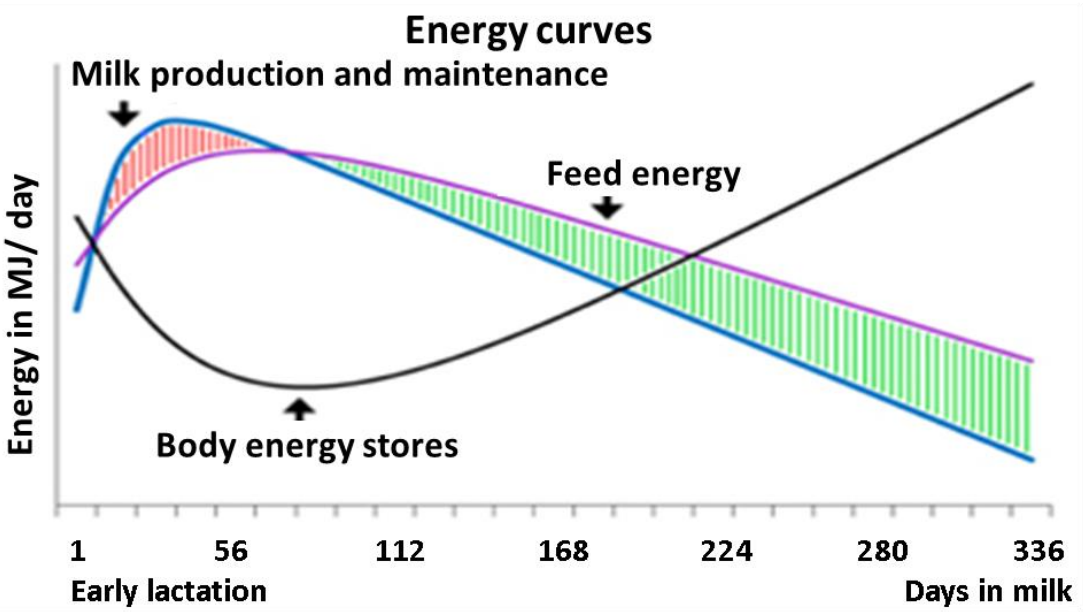

Figure 1.1: Energy supply and requirements during the lactation cycle of 340 days (Strucken et al. 2015).The blue curve represents the energy needed for milk production and maintenance of vital body functions. The energy needed for milk production is highest when milk

production reaches a peak. At the same time, the energy taken in through food (purple curve) cannot cover the energy requirements for milk production, which leads to a loss in body energy stores (black curve). This imbalance in energy homeostasis changes with the decline of milk production in late lactation.

A comparative study on the resumption of follicular growth early after calving in both beef and dairy cows documented to happen with the first dominant follicle detected morphologically after 10 days of post-calving (Crowe *et al.* 1998; Crowe *et al.* 2014). In this study, the first dominant follicle, and frequently a successive number of dominant follicles, generally fails to ovulate and rather undergo atresia in the case of dairy cows. With beef cows in good body condition, the first postpartum dominant follicle to ovulate is generally from wave 3.2 ± 0.2 8 (~30 days) whereas for beef cows in poor body condition there are typically 10.6 ± 1.2 waves of follicular growth before ovulation occurs ≈70 to 100 days (Crowe *et al.* 2014). In case of dairy cows' ovulation of the first post-calving dominant follicle typically occurs the same time in the 30% to 80% of cows, but generally undergoes atresia in 15% to 60% of cows or becomes cystic in 1% to 5% of cows (Crowe *et al.* 2014). In total, this NEB is associated with unwanted peripartum complications including suppression of dry matter intake, immunosuppression, increased peripartum complications and consequent infertility problems and decreased milk production (Wankhade *et al.* 2017).

1.1.4.1 Metabolic change and lipolysis during negative energy balance

In dairy cows, most of the physiological changes in metabolic and infectious diseases occurred during the transition period between 3 weeks peripartum and 3 weeks postpartum (Wankhade *et al.* 2017). Increasing body condition score during late pregnancy is a major factor causing decreased dry matter intake during peripartum (Butler 2003). The dry matter intake is reduced as high as by 28 % during the transition period peripartum (Bertics *et al.*

1992). This causes the onset of severe NEB during the first 2-3 weeks of postpartum with the onset of lactation (Butler 2003). After parturition, the rapid body weight loss and body condition score losses are the most obvious clinical symptoms of forced lipid mobilization due to NEB in early lactation (Huszenicza *et al.* 2004). This is due to the high demand for energy and nutrients for the synthesis of colostrum and milk in transition cows which leads them to undergo negative energy balance and micronutrient deficiencies (Wankhade *et al.* 2017).

During the period of NEB, animals have a shortage of glucose levels in their blood and start to compensate from other sources. Nutrient demands in late pregnancy increase insulin resistance in adipose tissue and muscle, coupled with increased sensitivity to lipolysis agents which reduce peripheral glucose uptake and facilitate nutrient flow away from maternal stores to the placenta (Savio *et al.* 1990). The utilization of peripheral tissue is an alternative source of energy during the high milk production period to compensate for the shortage of glucose availability by using lipolysis and ketogenesis from adipose tissue. As a result, cows starts to mobilize reserved fat which in turn increased blood plasma concentration of non-esterified fatty acids (NEFA) (van Knegsel *et al.* 2007; Fatima *et al.* 2014; O'Doherty *et al.* 2014). Elevated NEFA concentration is an indicator of excessive amounts of fat mobilization and that are associated with poor liver function. A study showed that a NEFA concentration of ≥0.3 to 0.5 mmol/L during the peripartum and dramatically increase to ≥0.7 to 1.0 mmol/L during the early postpartum period (Wankhade *et al.* 2017). Moreover, 50% of post-calving cows have reported suffering from temporary subclinical ketosis, which leads to an increase the concentration of beta-hydroxybutyrate (BHB) in the blood (Wathes *et al.* 2007).

The presence of high BHO, one of the ketone bodies a by-product of incomplete metabolism of fat, is an indicator of clinical and subclinical ketosis. Cows with blood BOH concentration

0.6 to 0.8 mmol/L during peripartum and 1.0 - 1.4 mmol/L during post-calving are considered as metabolically stressed one (Wankhade *et al.* 2017). Simultaneously, the accumulation of high lipids and triacylglicerols in the liver has been documented to causes fatty liver disease and resulted in a disorder in hepatocellular functions (gluconeogenesis, cholesterol, bile acid, and bilirubin metabolism, inactivation of steroid hormones and insulin) (Huszenicza *et al.* 2004b). Even though those changes are believed to be adaptive processes, cows failed to adapt to this metabolic stress and face severe metabolic and infectious disorders which causes the productive and reproductive inefficiency and increase during the transition period (Wankhade *et al.* 2017).

As a consequence, the ovarian follicle development in these postpartum cows occurs in a compromised endogenous metabolic environment as energy supply is prioritized towards the mammary tissues for milk production (van Knegsel *et al.* 2007b; Fatima *et al.* 2014; O'Doherty *et al.* 2014). Therefore, cows during NEB are documented to have a poor expression of oestrus, defective oocytes/embryos and uterine infections (Dobson *et al.* 2007). This leads to increased time laps to first ovulation and first breeding (McGuire *et al.* 2004). As a result, the calving rate of the modern dairy cow is reported to declining by 1% per year and first service conception rates by 40% (Royal *et al.* 2000). Furthermore, cows in the state of negative energy balance have high concentration with a threshold of NEFA > 0.55 mmol/L (Fenwick *et al.* 2008)' and ß-OHB > 0.65 mmol/L (Girard *et al.* 2015). The difference in the level of those blood metabolites could be associated with the breed and other related management practices. Follicular fluid and blood serum metabolite profiling in metabolically stressed postpartum cows has been documented as all metabolites change in serum to be reflected a similar pattern in the follicular fluid (Leroy *et al.* 2004). In the same study the measurement of glucose, b-hydroxybutyrate, urea, total protein and triglyceride, total

cholesterol and NEFA from day 14-46 in follicular fluid were documented to have similar pattern in both follicular fluid and blood serum.

Previous study showed that metabolite concentrations in follicular fluid were speculated to explain the differences in fertility between heifers and lactating cows (Bender et al. 2010). Characterization of follicular fluid from first wave dominant follicle and serum metabolites in dairy cows with similar genetic merit for milk production between the extremes good and poor genetic merit for fertility reported that, the abundance fatty acids (arachidic acid, heneicosanoic acid, myristic acid, behenic acid, myristoleic acid, heptadecenoic acid, cis-11-eicosanoic acid, nervonic acid, and g-linolenic acid) in follicular fluid was affected by genotype (Moore et al. 2017). Metabolite profiling of fatty acids myristic acid analysis concentration in follicular fluid preovulatory follicle has reported lower in heifers (0.4) and non-lactating cows (0.48) whereas significantly higher in lactating cows (0.62) and this amount was reported to significantly correlated with circulating concentrations of IGF1 in blood serum (Forde *et al.* 2015). Furthermore, IGF1 in blood serum was reported to moderately correlated with the overall percentage of cis-10-heptadecanoic acid (0.55), linolenic acid (0.60) and arachidonic acid (0.51) in follicular fluid (Forde *et al.* 2015). Moreover, the serum concentrations of non-esterified fatty acids and b-hydroxybutyrate have shown to be higher, while glucose, insulin, and insulin-like growth factor-1 concentrations were lower in lactating cows compared to non-lactating cows and heifers. Therefore, although the resumption and growth of the first dominant follicle and its successive number of dominant follicles morphological detection is as early as 10 days of post-calving and frequently fails to ovulate and rather undergo atresia (Crowe *et al.* 1998, Crowe *et al.* 2014).

1.1.4.2 Immunological change during negative energy balance

Negative energy balance is also associated with immunological suppression and a prolonged time for uterine involution. The major changes in the housing, feeding management, increased quality of roughage and increased on the proportion of concentrates have improved the production and efficiency of milk but not the disease incidence rates (Ingvartsen & Moyes 2013). As a consequence, during the transition period (3 weeks Peripartum and 3 weeks postpartum) significant change in metabolism is the most challenging period for dairy cows and other mammals that may threaten both health and welfare coping mechanisms associated with an elevated incidence of metabolic and other non-infectious and infectious disease including fatty liver, milk fever, retained placenta, metritis, ketosis, left-displaced abomasum, lameness and clinical mastitis (Ingvartsen & Moyes 2013).

In dairy cows uterus involution is a normally septic process; thus, more than 90% of cows have microorganisms in their uterus for the first 2 weeks following calving, 78% of them up to 16 -30 days, 50% of them up to 31 – 45 days, and 9% of them have until day 60 of post-calving (Ghanem *et al.* 2015). During sever and mild negative energy balance, the concentrations of circulating IGF-I and glucose have been documented to lower during early post-calving (Fenwick *et al.* 2008). Enough concentration of glucose is a key source of energy by phagocytic cells, proliferation, survival and differentiation and glucose preferred metabolic fuel during inflammation for macrophages and lymphocytes rather than fatty acids, amino acids or ketone bodies (Ingvartsen & Moyes 2013). Therefore, during the low availability of glucose, a bacterial infection is common during early post-calving in lactating dairy cows. In the first two weeks of post-calving, positive endometrial cultures have been reported to be associated with infection of one or more from A. pyogenes, E. coli, Pseudomonas spp., Streptococcus spp., Staphylococcus spp., Pasteurella multocida, Clostridium spp., Fusobacterium spp. and Bacteroides species (Esposito *et al.* 2014). A study on 5,719 dairy cows at day 65 of post-calving showed that cows were clinically diagnosed with metritis

(37.8%), clinical endometritis (38.7%), fever (39.8%), mastitis (39.4%) and only 51.4% were healthy cows (Santos *et al.* 2010). Surprisingly, dry matter intake was reported to reduce in cows with severe metritis by 2–6 kg DM compared to healthy cows in the 2–3 weeks of post clinical signs of metritis (Esposito *et al.* 2014). In addition to that, cows with uterine bacterial infections had been reported to have a prolonged luteal phase relatively from cows without infection (Ghanem *et al.* 2015). Furthermore, uterine infection and endometritis have been reported to be correlated with lower artificial insemination successes (Wankhade *et al.* 2017). Currently, immunological problems in dairy cows are contributing to reproductive inefficiency, culling, treatment costs, milk discard, labor and increased risk of residues in food products (Esposito *et al.* 2014).

1.1.4.3 Effect of metabolic stress on the endocrine profile

Negative energy balance is known to affect the level of different reproduction hormones in the blood circulation. Progesterone hormone is essential for pregnancy after artificial insemination and must be present in the blood to support embryo development and survival. Furthermore, biochemical characterization of progesterone showed to have a stabilizing effect on amino acid flux which is important to conceptus elongation initiation include arginine, fructose, glutamate, and mannitol (Simintiras *et al.* 2019). In lactating cows with NEB have, the concentration of plasma progesterone was reported to decline by 25–50% compared to heifers (Huszenicza *et al.* 2004). The concentrations of progesterone and oestradiol in plasma during post-calving anestrus were documented to be as low as 0.2 ng/ ml and (<5 pg/ ml respectively (Savio *et al.* 1990). The production of blood plasma FSH has been reported to be reduced for a few days before and after parturition and in most cows plasma FSH levels increased as early as day 5 post-calving indicating limiting factor to the resumption of ovarian cyclicity after calving (Opsomer *et al.* 1996). Insulin-like growth factor I concentrations in serum and FF of dairy cows were also reported to positively correlated with energy status and

are essential for normal follicular development (Leroy *et al.* 2008c). Furthermore, the pattern of LH secretion in postpartum cows is reported to be detected during first week of post-calving with a frequency as low as 2-3 pules per 6-hr period at day 5 of post-calving and increased to 5-7 pulses per 6-h period in the presence of a dominant or cystic follicle (Savio *et al.* 1990). This hormonal imbalance in post-calving cows leads to delay in ovulation and folliculogenesis beyond the normal transition period for breeding.

1.1.4.4 Effect of negative energy balance on oocyte maturation and early embryo development

In order to make the appropriate developmental decisions or maintain homeostasis, cells and organisms must coordinate the expression of their genome and metabolic state (van der Knaap & Verrijzer 2016). The metabolic environment of the mother in which the fetus is growing has an impact on the metabolism of the upcoming offspring and which could be reflected in changes in developmental programming including changes in epigenetic marks (DNA methylation or histone tail modifications (Vickers 2014). Various *in vitro* studies involving the supplementation of NEFA on oocyte development evidenced the negative effect of NEB during early development. Elevated NEFA concentrations supplementation during IVF for both sperm and oocyte have been documented with a major effect on oocyte except relative reduction on sperm motility and plasma membrane integrity (Desmet *et al.* 2018).

It has been suggested that lipolysis conditions believed to affect the fertilization and blastocyst rate (Desmet *et al.* 2018). Moreover, supplementation of NEFA during bovine oocytes and embryos culture has a profound effect on gene expression and methylation patterns (Desmet *et al.* 2016). Moreover, sever negative energy balance has been reported to induce the expression of genes involved in lipid catabolism (e.g. acyl-coenzyme A

dehydrogenase), gluconeogenesis and downregulation of genes involved in insulin-like growth factor (Wathes *et al.* 2007).

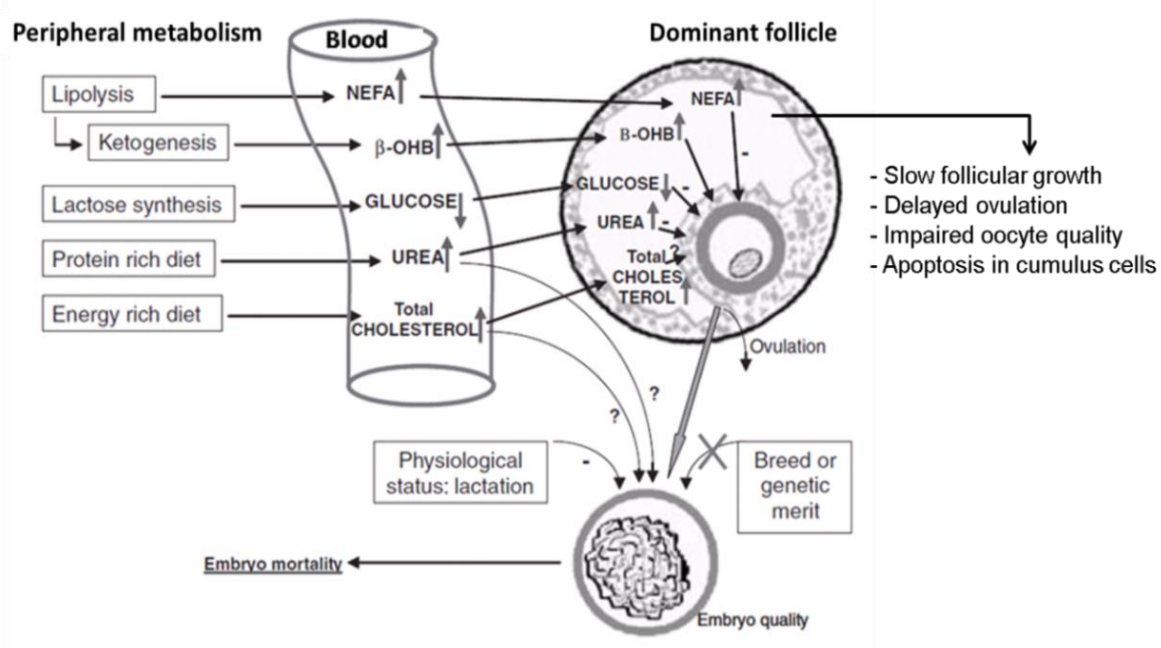

Figure 1.2: The effect of negative energy balance on oocyte maturation and possible mechanisms by which embryo quality can be impaired in high-yielding dairy cows adopted from (Leroy *et al.* 2008a)

After fertilization, the early embryo resides in the oviduct for 4 days during which the embryo is highly sensitive to environmental changes (Leroy *et al.* 2008). Inadequate corpus luteum function, associated with reduced progesterone, and low insulin-like growth factor concentrations cause a suboptimal microenvironment in the uterus that is incapable of sustaining early embryonic life (Leroy *et al.* 2008c). Supplementation NEFA (palmitic acid and stearic acid) on the culture medium with elevated concentrations during oocyte maturation has reported to significantly reduce the progression of meiosis, and the subsequent fertilization, cleavage rates and blastocyst formation (Leroy *et al.* 2005). Sever NEB at day 14 of post-calving has been shown to affect liver gene expression in which hepatic cows displayed reduced expression of transcription activators and signal transducers that regulate

the expression of genes and gene networks associated with cell signaling and tissue repair (McCarthy *et al.* 2010).

Furthermore, sever negative energy balance in postpartum cows has been found to affect liver miRNA expression and these miRNAs had putative targets in hepatic genes that are down-regulated under this condition (Fatima *et al.* 2014). The endocrine and biochemical changes, which are associated with NEB, are reflected in the microenvironment of the growing and the maturing female gamete, and likely result in ovulation of a developmentally incompetent oocyte (Leroy *et al.* 2008c). Supplementation of elevated concentration of NEFA has been reported to be toxic in bovine granulosa cell growth and function in vitro (Leroy *et al.* 2005). Therefore, during NEB with a low concentration of glucose, the follicular cells obliged to use alternative sources of energy and various biomolecules in the follicular fluid such as ketone bodies, lipids, amino acids, lactate, pyruvate, and others are excessively available. Adequate managerial measures have been recommended to reduce the duration as well as the depth of the NEB and the consequent effect on endocrine and metabolic functions in general, and on oocyte and embryo quality in particular (Leroy et al. 2008b).

1.1.5 Follicular fluid composition and oocyte metabolism

Oocyte growth and development are highly dependent on the nurturing environment of the follicular fluid to reach its developmental competency and resulted in an incompetent embryo. Follicular fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and of the secretory activity of granulosa and theca cells and provides a very important microenvironment for the development of oocytes (Revelli *et al.* 2009). Nutritionally, it is composed of locally produced substances that are related to the metabolic activity of ovarian cells (Gerard *et al.* 2002). The chemical composition of follicular fluid from the dominant follicles is important because it is an indicator of the secretory activities

and metabolism of follicular cells and thus could be related to the follicular quality (Revelli *et al.* 2009).

Differences in the metabolite composition of follicular fluid were correlated with the developmental competence of human oocyte and predicted to be used as an important technique in gamete and embryo selection (Wallace *et al.* 2012). Even though, the oocytes have a low capacity for glucose metabolism, the cumulus cells with high glycolytic activity are able to metabolize glucose into pyruvate, which transfers into the oocyte as an energy source during oocyte maturation (Dumesic *et al.* 2015). Furthermore, oocyte quality is associated with follicular fluid metabolic analysis in polycystic ovary syndrome in human (Arya *et al.* 2012). Follicular fluid metabolite analysis from the subordinate follicle has reported having higher in NEFA (palmitoleic acid and palmitic acid) compared to dominant follicles (Bender *et al.* 2010). Endocrine-disrupting chemicals contamination in the follicular fluid was reported to be associated with the fertilization rate, oocyte developmental competence, and quality of an embryo, and the endocrine-disrupting chemicals concentrations in blood serum were reported to be reliable predictors of the contamination status of the follicular microenvironment (Petro *et al.* 2012).

1.1.6 Follicular fluid-based diagnosis of oocyte and early embryo outcome

As the demand for in vitro fertilization and embryo transfer increased in the globe, selecting the best embryo remains the hallmark to improve efficiency. With this regard, a preimplantation genetic diagnosis is a useful tool for genetic evaluation of embryos, however, the technique remains limited applicability due to cost, availability, inaccurate results, incomplete genetic evaluation, and other factors (Kovalevsky & Patrizio 2005). The current embryo quality assessment is dependent on different parameters such as the oocyte competence, fertilizability, morphology, and cleavage rate. These types of embryo quality analyses are mostly less reliable, invasive and time-consuming, and required highly skilled

manpower. Consequently, the quality assessment of an embryo has been not fully understood yet regardless of the high success rate in IVF. The vast majority of embryos produced in vitro and transferred to recipient cows fail to develop to term as only less than 30% resulted in a live birth in human (Kovalevsky & Patrizio 2005).

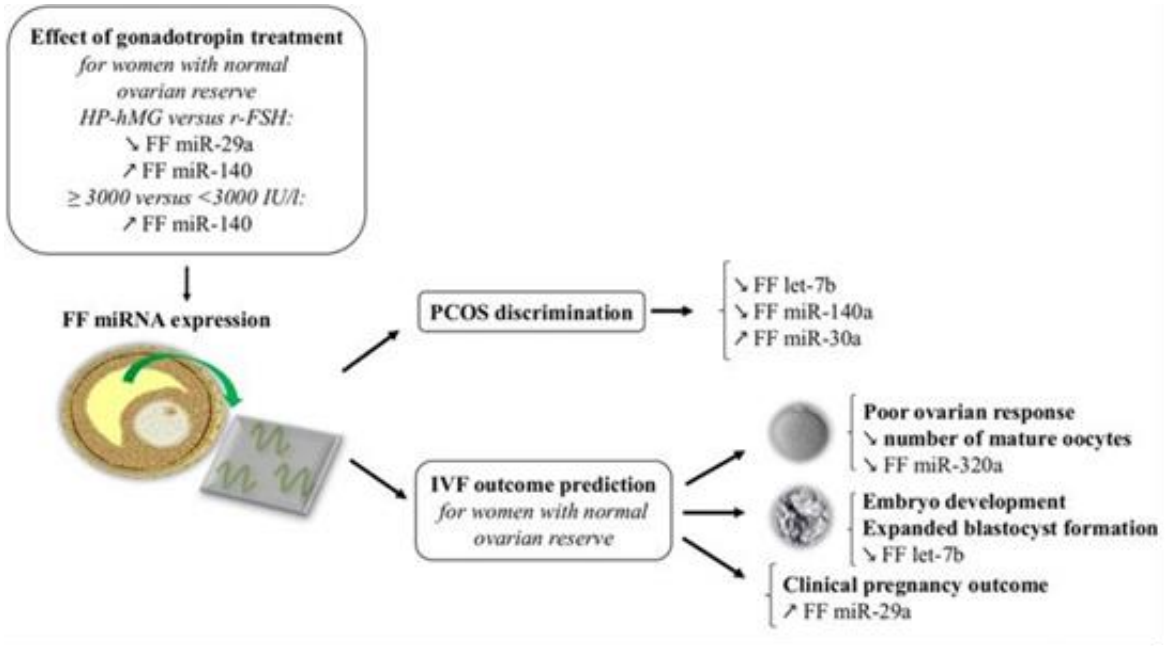

Figure 1.3: Schematic model showing that miRNA expression profiling in follicular fluid as tools to efficiently discriminate and predicted IVF outcome in women with polycystic ovary syndrome (Scalici *et al.* 2016).

Advancements in the single-embryo transfer would be an effective way of reducing multiple gestation rates, but its widespread acceptance is limited because of the inaccurate method of embryo assessment and selection (Wallace *et al.* 2012). For the last three decades oocyte and embryo, quality assessment is a key mediator of assisted reproductive technology. The quality assessment procedures for oocyte and embryo are mostly dependent on the morphological characterization of the oocyte, polar body extrusion, embryo cleavage, the number of blastomeres. A number of technologies are emerged including; assessment of glucose, lactate, pyruvate, and amino acid metabolism, proteomic profiling, evaluation of oxygen

consumption, and most recently, the examination of the metabolites (Bromer & Seli 2008). Follicular fluid could be the next alternative reliable source of biomarkers and could potentially use as supplemental prognostic/diagnostic tools in ART. A previous study showed that the use of metabolic analysis of follicular fluid could help as an indicator of positive and negative pregnancy outcomes (Wallace *et al.* 2012).

Most surprisingly, the implementation of cell-free DNA evaluation in individual follicular fluid samples might represent an innovative biomarker of embryo quality to use as a supplemental tool to predict embryo quality during IVF (Scalici *et al.* 2014). A study on the detection of follicular fluid circulatory nucleotide reported the presence of DNAs (Scalici *et al.* 2014) and miRNAs (Sohel *et al.* 2013). Furthermore, miRNA expression profiling in follicular fluid was reported to be a potential tool to efficiently discriminate and predicted IVF outcomes in women with polycystic ovary syndrome (Scalici *et al.* 2016).

1.1.7 The morphological and physiological changes during folliculogenesis

In the mammalian reproductive process, the ovaries have a huge number of primordial follicles consisting of their individual oocyte arrested in prophase-I of meiosis covered by a single layer of granulosa cells. Those primordial follicles are continuous development during the foetal life in primates and ruminants, or they develop during the early neonatal period in rodents and rabbits (Fortune 1994). In human ovary, greater than 99% of the follicles present at birth are destined to atresia during life in which less than 400 out of 400,000 follicles found at puberty eventually to ovulate(Tilly *et al.* 1991). In bovine, during each follicular wave, a cohort of follicles are initiated to grow beyond 4 mm in diameter and only a single follicle is selected (LH) and LH surge is highly detected only in healthy follicles with a diameter in > 9 mm in diameter (Xu *et al.* 1995).

The two major functions of the mammalian ovary are the production of oocytes which is going to allow continuation of the species, and generation of bioactive molecules including estrogens, progestins, peptide and growth factors critical for ovarian function, regulation of the hypothalamic-pituitary-ovarian axis, and development of secondary sex characteristics (Edson *et al.* 2009).

In general, follicle development in mammals is dividing into two phases; the preantral a gonadotropin-independent phase, which regulated by autocrine and paracrine signals and the second phase of follicle development, is the gonadotropin-dependent and characterized by the rapid proliferation and secretive activity of granulosa cells. After puberty, the follicle starts gradually and continually leaving the resting pool and starts growth although the nature of the signals that initiate growth and the mechanisms of that follicles are unknown (Fortune 1994). Once activated, the physiology of folliculogenesis has been confirmed to take almost a year from recruitment to growth in human (Erickson & Shimasaki 2001).

Folliculogenesis is consists of the preantral phase (gonadotropin-independent), characterized by the growth and differentiation of the oocyte and controlled by locally produced growth factors through autocrine/paracrine mechanisms (50 days), and the antrum phase (gonadotropin-dependent), characterized by the tremendous increase of the size of the follicle itself and regulated by FSH and LH as well as by growth factors of 300 days (Erickson & Shimasaki 2001). In bovine, the period from recruitment to growth takes 100 days to produce the dominant follicle from the primordial follicle (Britt 2008). After the development of a preovulatory follicle, the early antrum stage to ovulation is estimated to be 40days (Llewellyn *et al.* 2007).

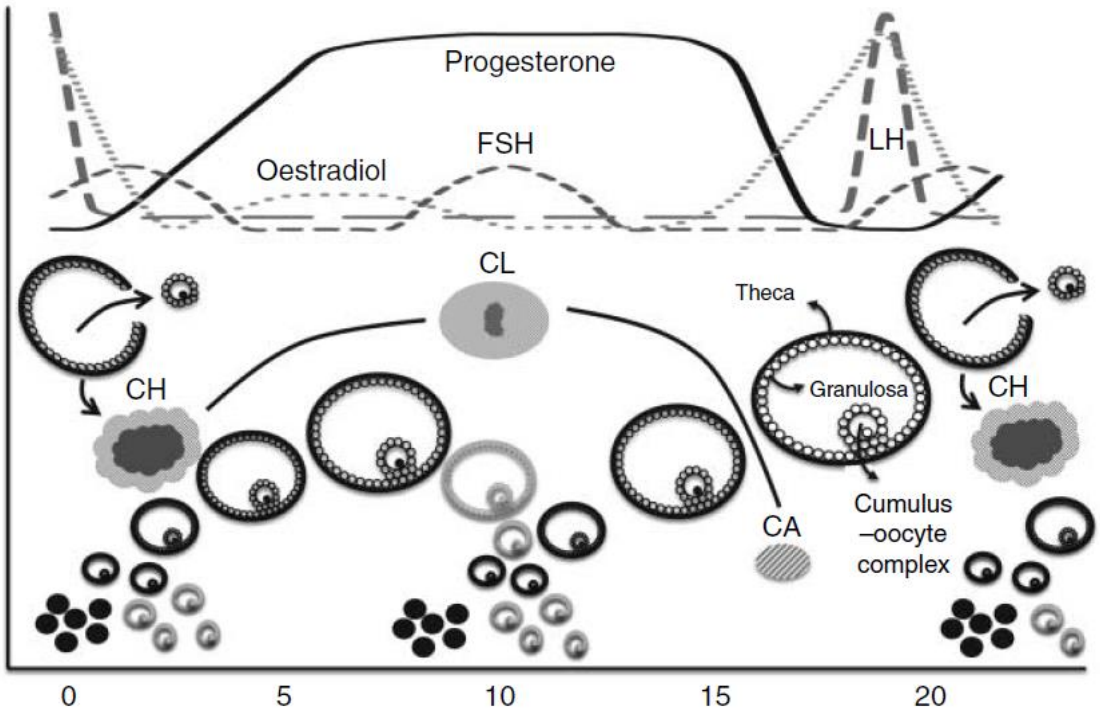

Figure 1.4: Diagram on the endocrine and paracrine regulation of follicular development in bovine: (FSH: Follicle stimulating hormone; CL: corpus luteum, corpus lethum, CA: corpus albicans, CH: corpus haemorrhagicum) (Donadeu *et al.* 2012).

Follicular selection in monovular species such as human, bovine, and horse is a common phenomenon in which only one follicle develops from a wave of growing follicles and continues to grow in diameter and ovulates, whereas the other follicles remain to be subordinate follicles (Beg & Ginther 2006). In cattle, waves of follicular activity occur periodically and the dominant follicle emerges every 10 days, and each wave contributes as large as 10 mm to the dominant follicle population (Ginther *et al.* 1989). Therefore, two or three large follicles develop during consecutive waves of follicular growth within an oestrous cycle. The last wave provides the ovulatory follicle, whereas the preceding wave provides follicles that undergo atresia depending on the secretion of LH (Lucy et al. 1992).

At the final stage of oocyte maturation, follicular fluid content of oestradiol, progesterone, and testosterone had been studied using radioimmunoassay and micro-morphologically by

Dieleman *et al.* (1983). During this stage, steroidogenesis plays a role in producing estradiol and divided into three phases. During the first phase (0–6 hrs after the LH peak) oestradiol remained constant at a lower level whereas the progesterone concentration and the size of theca internal cells increased by 40%. During, the second phase (6-20 hrs after the LH peak), the level of oestradiol and progesterone dropped rapidly. However, in this phase, the width of the theca interna cells decreased in size to that of preovulatory follicles whereas the granulosa cells increase slightly. In the third phase (20 hrs after the LH peak until ovulation), the level of oestradiol decreased further, and progesterone increased to the highest levels whereas the size of the granulosa cells increased by 39% and the width of the theca interna cells remained the same.

The second change that happened during the final stage of oocyte maturation is morphological changes in the follicular wall. Oocyte maturation has three different developmental stages. Specifically; 1) a germinal vesicle stage of the oocyte and characterized by a compact granulosa cell layer and high mitotic activity; 2) the mid maturational stage the oocytes which is characterized by a follicular wall with expanded granulosa cell layer with little mitotic activity; and 3) a metaphase II (MII) stage: is characterized by a highly expanded granulosa cell layer with no or very little mitotic activity and the presence of eosinophylic granulocytes outside the vascular compartment, invading the granulosa cell layer (Loos *et al.* 1991; Dieleman & Bevers 1993). The MII phase of oocyte maturation is occupied by cytoplasmic and nuclear maturation. During this period, 6 hrs after the LH peak, resumption of meiosis and synthesis of mRNA is necessary for the oocyte to undergo GVBD and for further nuclear progression to the M-I1 stage followed by protein synthesis (Dieleman & Bevers 1993). Oocyte nuclear maturation is characterized by a meiotic resumption of a competent oocyte at a diameter of least 110 µm in bovine (Sirard *et al.* 1998). The summary

of the endocrine and paracrine regulation of follicular development in bovine as indicated in Figure 1.4.

1.1.8 Intracellular communication of the oocyte and the surrounding somatic cells

Cell-to-cell communication during folliculogenesis is a sine qua non between the oocyte and its corresponding somatic in the follicular microenvironment. At birth, the ovaries of mammalian females contain a finite store of the primordial follicle of the cumulus-oocyte complex. The communication system between the oocyte and surrounding folliculogenesis cells is through the gap junction network to facilitates the transfer of signals and nutrients in-to and out-off the oocyte and the follicle cells (Fair 2003). Oocyte growth is a gap junction dependent in which the oocytes and the surrounding granulosa cells extend cellular processes towards one another and establish bidirectional communication (Amleh & Dean 2002). During folliculogenesis, bidirectional communication between the growing oocyte and neighboring somatic cells is crucial for oocyte developmental competence (Gilchrist *et al.* 2004).

The cellular differentiation that occurs during follicular development is tightly regulated by the expression and interaction of multiple genes in a spatiotemporal manner in different compartments of the follicle: granulosa cells, theca cells, follicular fluid, and oocyte. Deletion of growth differentiation factor-9 (GDF9) in oocyte has demonstrated to cause a block in follicular development and infertility in mice (Dong *et al.* 1996) and confirmed GDF9 as a proliferation factor for granulosa cells (Vitt *et al.* 2000). The presence or absence of oocyte and cumulus cell factors during bovine oocyte maturation have been observed to cause a profound effect on transcript abundance of each cell type showing the prevailing molecular cross-talk between oocytes and their corresponding cumulus cells in bovine (Regassa *et al.* 2011). The presence or absence of oocytes or cumulus cells during maturation was found to affect the expression of miRNAs in each of the two cell types (Abd El Naby et al. 2013).

1.1.9 Molecular mechanism of oocyte and embryo developmental competency

In many species, the mammalian development process begins during oogenesis when maternal RNAs and proteins have accumulated during oocyte growth. Early embryonic development is primarily reliant on maternal transcripts synthesized during gametogenesis (Misirlioglu *et al.* 2006). Initially, maternal proteins and RNAs are known to support development, whereas a number of zygotic and embryonic genes are expressed in a stage-specific manner following embryonic genome activation (Bilodeau-Goeseels & Schultz 1997). In cattle, the maternal-to-embryonic transition is characterized by a minor gene activation between the 1- and 4-cell stages with a major gene activation after 8- to 16-cell stages (Memili & First, 1999). Global embryonic genome activation is the most critical event of bovine preimplantation embryogenesis, which consists of dividing the cytoplasm, changing the length of cell cycles, and replicating DNA until the first cell differentiation occurs (Misirlioglu *et al.* 2006). Gene expression during early mammalian development divided into two major phases. The first phase is characterized by dependence on maternal macromolecules inherited from the oocyte and the second is characterized by activation of the new gene from the embryonic genome (Leibfiied-Rutledge, 1996).

In mammals, gene regulation plays a crucial role in the development and fulfilled by the rapid shifts in molecular programs that occur through transcription regulation, mRNA stability, protein translation, protein stability, and protein activity. Post-transcriptional regulation of gene expression has been documented to be much more intricate than previously thought and elucidating the basic mechanisms of post-transcriptional control has been speculated to be essential to gain a full understanding of how gene expression is regulated at different levels including posttranscriptional dysfunction of numerous genetic disorders and cancer. The discovery of small noncoding RNA; microRNA increased the understanding of the

posttranscriptional gene regulation mechanism and change the actual dogma of molecular biology.

1.1.10 The function of miRNAs during folliculogenesis

MicroRNAs (miRNAs) are small non-coding RNAs (~22 nucleotides), which play a vital role in gene regulation at the post-transcriptional level in multi-cellular and unicellular organisms in a spatiotemporal manner. MicroRNAs were discovered for the first time in C. elegance, when lin-4 transcripts were found to contain sequences complementary to in the 3' untranslated region of lin-14 mRNA and regulate translation via an antisense RNA-RNA interaction (Lee *et al.* 1993). Nearly 0.5-1 % of the genes in the genome encode for miRNAs which may regulate up to 30% of the genes in human (Lim *et al.* 2003; Lewis *et al.* 2003). Mammalian miRNAs exert their gene regulation mechanism through mRNA degradation, translational repression and translational initiation (Valencia-Sanchez *et al.* 2006; Behm-Ansmant *et al.* 2006; Vasudevan *et al.* 2007). Several studies showed that, miRNA mutations, biogenesis defects or deregulation can affect miRNA-mediated gene silencing, and subsequently diseases conditions including cancers, cardiovascular diseases, neurological disorders, ischemia, heart failure, hepatitis, sepsis, tuberculosis, diabetes, and obesity (Traver *et al.* 2014).

In many species including cattle, global expression of miRNAs showed the essential roles of miRNA in regulating maternal mRNAs and supporting early embryonic development. Impaired biogenesis of global miRNA expression had demonstrated to cause disturbance oocyte and embryonic development. Deletion of Dicer in mice during early development demonstrated to cause blockage of differentiation processes at the blastocyst stage (Cui *et al.* 2007). A comparison of microarray miRNAs profiles of mice control and mutant oocytes have reported that a large proportion of the maternal genes are directly or indirectly under the control of maternal miRNAs are essential for the earliest stages of mouse embryonic

development (Tang *et al.* 2007). Dicer is essential for turnover of a substantial subset of maternal transcripts that are normally lost during oocyte maturation and dicer knockdown mice oocyte have reported chromosomal congression defects in meiotic progression, cleavage and fertilization (Murchison *et al.* 2007) and embryonic lethal at day 7.5 (Bernstein *et al.* 2003). Adult female null dicer mice have reported showing normal mating behavior but failed to produce an offspring due to oviductal defect (Hong *et al.* 2008). Abnormal embryonic morphogenesis in zebrafish (Wienholds et al. 2003), and abnormal morphogenesis during gastrulation, brain formation and heart development (Giraldez *et al.* 2005).

Different holistic approaches to study miRNAs expression and their knockdown experiments in different species have evidenced the vital role of miRNAs in the posttranscriptional gene regulation mechanism. Genes found either specifically or predominantly in the oocyte are likely regulated by miRNA (Tripurani *et al.* 2010). Moreover, impaired biogenesis of miRNA has been demonstrated to cause disturbance of chromosomal alignment, embryonic lethal and infertility in mice early development (Murchison *et al.* 2007). In addition, miRNAs have documented to be important players in the maternal-to-embryonic transition, as expression profile suggested a potential regulatory role during early development stages. The presence of a huge number of miRNAs have been confirmed in the ovary (Hossain *et al.* 2009), oocyte and cumulus cell (Tripurani *et al.* 2010, Abd El Naby *et al.* 2013) and granulosa cell (Salilew-Wondim *et al.* 2014; Gebremedhn *et al.* 2015) and reported to target various genes.

Different functional study experiments on several individual miRNAs have revealed the importance of miRNA during oocyte maturation and early development. For example, inhibition of miRNA-21 in mice during early development reported by targeting the expression of yet unknown gene transcripts to maintain granulosa cell survival and promote ovulation (Christenson 2010). Moreover, a significant rise in the expression of miR-21 and miR-130a from 1 to 8 cells has been suggested as an indicator of the activity of miRNAs on

mRNAs carrying a seed sequence in their 3′UTR during the first segmentations of the bovine embryo (Mondou *et al.* 2012). Poor response to in vitro fertilization has been reported to be associated with altered miRNA expression in cumulus cells specifically with elevated expression of miR-21-5p independent of lower serum (Karakaya *et al.* 2015). The summary of biogenesis and the function of miRNAs their machinery genes (Fig 1.5).

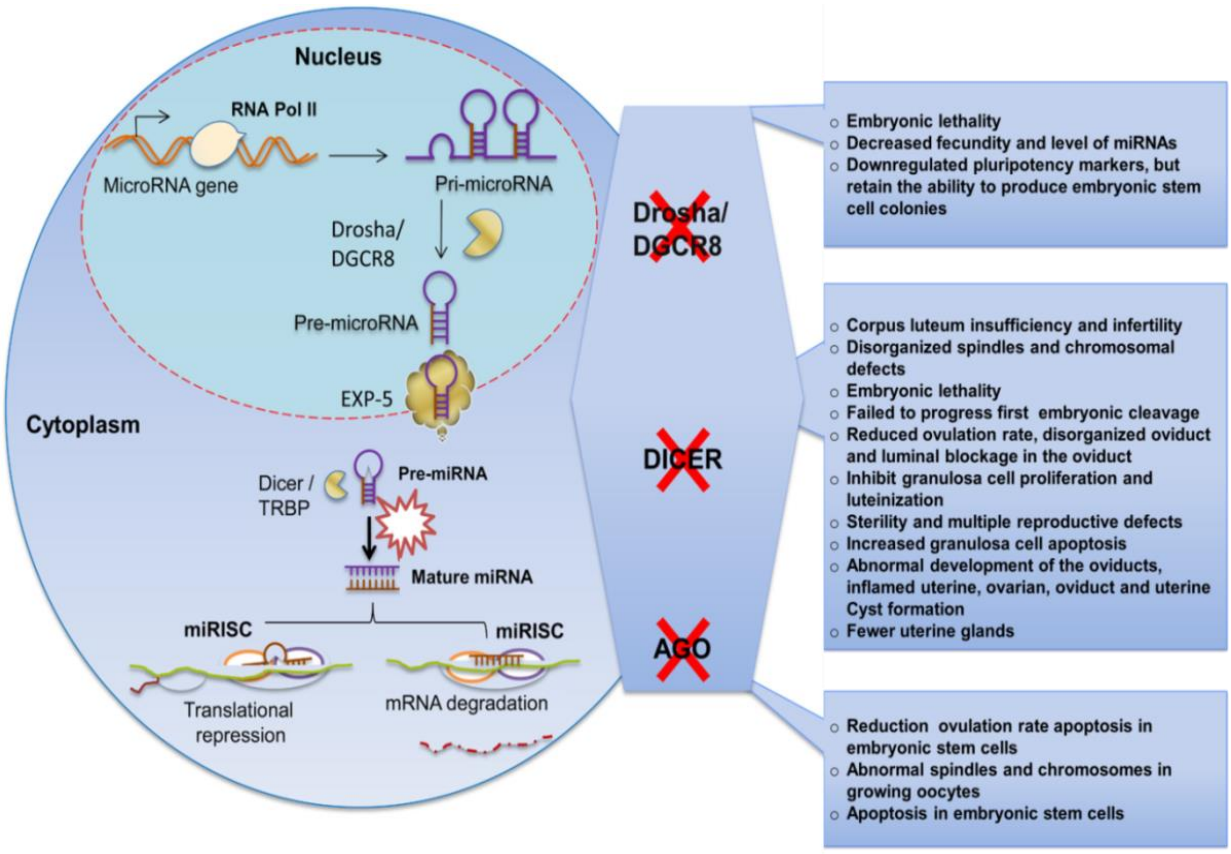

Figure 1.5: Summary of microRNA biogenesis, posttranscriptional gene regulation mechanism and selective knockout of key miRNA machinery genes associated phenotypic defect on mammalian female fertility (Tesfaye *et al.* 2018).

Recently, circulating miRNAs have been suggested as biomarkers of specific conditions because they are relatively abundant and stable due to their confinement within extracellular vesicles where they are protected from RNases (Traver *et al.* 2014). MicroRNAs have found actively secreted from cells and packaged into appropriate carrier extracellular vesicles.

Afterward, the extracellular vesicles are able to diffuse into the extracellular space microenvironment of the body and served as a means of communication with other cells or tissues. This opens the new dimension of cell- to- cell communication beyond previously known methods.

1.1.11 Extracellular vesicle-mediated cell-to-cell communication

Intercellular communication is an essential hallmark of multicellular organisms and can be mediated through direct cell–to–cell contact or transfer of secreted molecules (Raposo & Stoorvogel 2013). Cell-to–cell communication has proven to be even more complex than previously thought since the discovery that extracellular vesicles serve as carriers of biological information on various pathophysiological settings (Loyer *et al.* 2014). Extracellular vesicles are evolutionarily conserved communicasomes of most cells in all domains of life on earth from archaea, gram-negative and positive bacteria to eukaryotes (Choi *et al.* 2013, Kim *et al.* 2015). Cells release diverse types of membrane vesicles of endosomal and plasma membrane origin called exosomes and microvesicles into the extracellular environment (Raposo & Stoorvogel 2013). Exosomes were discovered for the first time in the Exfoliated membrane of a neoplastic cell line (Trams *et al.* 1981). This was followed by in vitro culture of sheep reticulocytes and speculated to have a physiologic function and suggested as vesicle externalization may be a mechanism for the shedding of specific membrane functions which are known to diminish during maturation of reticulocytes to erythrocytes (Johnstone *et al.* 1987).

Recently, studies reported that cells may also communicate with each other through extracellular vesicles as carriers of specific molecules and cells secrete a wide range of extracellular vesicles of different size, morphology, content, and function that interact with target cells and modify their phenotype and function (Kobayashi *et al.* 2015). Mammalian cells also secrete extracellular vesicles either constitutively or in a regulated manner as an

acellular tool for their intercellular communication (Choi *et al.* 2013). Extracellular vesicles (EVs) are classified into exosomes, microvesicles, and apoptotic bodies originating from different subcellular compartments (Loyer *et al.* 2014). These cargo-molecules have been reported to encompassing several different vesicle types, released by cells constitutively or in response to specific stimuli or cell stressors, including exosomes, microvesicles, apoptotic vesicles and in pathological situations, necrotic debris (Tannetta *et al.* 2014). During the early stages of the human reproductive process, the ovarian follicle, seminal fluid, endometrium, embryo, and trophoblast cells are all possible sources of EVs that have the potential to modulate maternal immune function locally (Tannetta *et al.* 2014).

Extracellular vesicles are the first biological cargo, specific and believed to be the "fingerprint" of the releasing cell and its metabolic status (Kobayashi *et al.* 2015). Exosomes are intraluminal vesicles range approximately from 30-100nm in diameter and secreted by live cells (Beach *et al.* 2014b) and have been documented to be found in healthy and pathological cells. Currently, exosomes have been found in almost all cellular fluids such as in nasal mucosal (Qiu *et al.* 2012a), cerebrospinal fluid (Saman *et al.* 2012b), breast milk (Admyre *et al.* 2007b) , saliva (Ogawa *et al.* 2008b), umbilical cord blood (Li *et al.* 2008), urine and amniotic fluid (Keller *et al.* 2007), follicular fluid (Sohel *et al.* 2013) and semen (Beach *et al.* 2014). Extracellular vesicles were documented in different species to carry different cargo-molecules including mRNA, miRNA, lipids, and proteins and could be translated into target cells. According to EXOCarta 2019, 9,769 of proteins, 3,408 of mRNAs, 2,838 of miRNA and 1,116 of Lipid have been documented (http://www.exocarta.org/).

1.1.12 Composition and biogenesis of extracellular vesicles

Based on the mechanisms of biogenesis, mammalian cells secrete two types of extracellular vesicles either constitutively or in a regulated manner: exosomes (50-100 nm in diameter) released from the intracellular compartment and ectosomes (also called microvesicles, 100-

1000 nm in diameter) shed directly from the plasma membrane (Choi *et al.* 2013, Cocucci & Meldolesi 2015, Sato-Kuwabara *et al.* 2015). These membrane vesicles are bilayer spherical structures enriched with various bioactive materials including proteins, genetic material (mRNA, microRNA, rRNA, and tRNA), and lipids (Choi *et al.* 2013). EVs also contain a large variety of other small noncoding RNA species, including RNA transcripts overlapping with protein-coding regions, repeat sequences, structural RNAs, tRNA fragments, vault RNA, Y RNA, and small interfering RNAs (Raposo & Stoorvogel 2013). Examples of tetraspanins commonly found in EVs are CD63, CD81, and CD9 (Colombo *et al.* 2014). Those different proteins are in fact present in some subtypes of EVs and not in others. For instance, histones and proteasome and ribosome components are probably secreted in large plasma membrane-derived EVs and/or apoptotic vesicles rather than exosomes (Colombo *et al.* 2014). Another tetraspanin was CD24 and suggested to be a marker of exosomes secreted into urine and amniotic fluid (Keller *et al.* 2007).

Living cells release different types of vesicles such as apoptotic bodies, microvesicles, exosomes, exosome-like and probably other types of particles. The production and the release of different types of extracellular vesicles are dependent on the physiological condition and the type of cells. Cells release different types of vesicles such including exosomes, microvesicles, apoptotic vesicles and in pathological situations, necrotic debris cargo-molecules either constitutively or in response to specific stimuli or cell stressors (Tannetta *et al.* 2014). Exosomes are formed from internalized endocytic vesicles and constitutively secreted from the cell (Tannetta *et al.* 2014) whereas ectosomes are ubiquitous vesicles assembled at and released from the plasma membrane (Cocucci & Meldolesi 2015). Microvesicles can be constitutive or require activating signals depending on the cell type and can be shed from normally functioning cells and/or at the beginning of the apoptotic process (Pap *et al.* 2009).

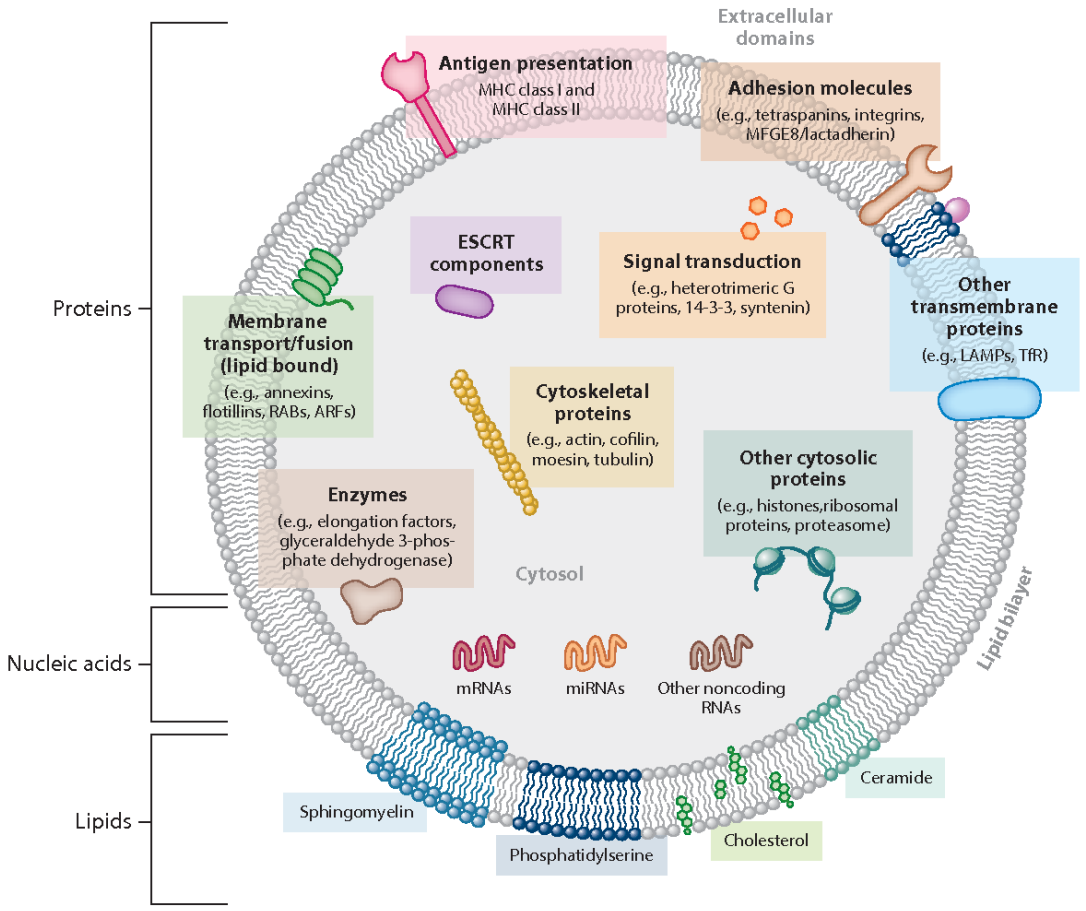

Figure 1.6: Overall composition of extracellular vesicles and schematic representative of their composition (Colombo *et al.* 2014)

The membrane of the microvesicles is consists of both lipids and proteins displaying some typical markers of the parental cell origin and the membrane molecular pattern and the internal content of the vesicle depends on the original cell type and on the mechanism of the release (Pap *et al.* 2009). Microvesicles are generally larger (up to ~1,000 nm in diameter), but also small vesicles (100 nm) may bud from the cell surface (Raposo & Stoorvogel 2013). Microvesicles are released from the plasma membrane through multiple pathways including the Ca2+ dependent manner in monocytes, in platelets, in red blood cells, in endothelial cells, in T cells, and in mast cells and the increase of intracellular Ca2+ level has been documented to change the asymmetric phospholipid distribution of the plasma membrane (Pap *et al.* 2009). Therefore, the increase in the cytoplasmic Ca2+ level results in the inhibition of

translocase and in the activation of scramblase, so that phosphatidylserine and phosphatidylethanolamine will not be returned to the inner side of the membrane. The other type of extracellular vesicles and the most studied once are exosomes an endosomal origin and different studies documented that they are smaller in size compared to microvesicles and the size ranges between30-100 nm. Exosomes are released via exocytosis when multivesicular bodies leave the lysosomal pathway and fuse with the plasma membrane (Pap *et al.* 2009; Raposo & Stoorvogel 2013).

1.1.13 Extracellular vesicle uptake and distribution

After the secretion, different types of extracellular vesicles could circulate in the local extracellular space and travel long distances by diffusion through body fluids, such as blood and uptaken by cells. EVs deliver complex signals to stimulate target cells by inducing receptor-mediated intracellular signal transduction by surface-expressed or bound ligands, transferring surface receptors to target cells, and delivering functional proteins, RNAs, and lipids into the target cells by fusion with the plasma membrane or internalization into the endocytic compartment (Choi *et al.* 2015). While a fraction of EVs is degraded by interactions with recipient cells and the rest are systemically cleared from the circulation *in vivo* as rapid as with a half-life of 2 minutes (Choi & Lee 2016) followed by slowly cleared from the liver, spleen, and lungs. The size of EVs is has been reported to be large to pass the endothelial cells (Choi & Lee 2016).

Extracellular vesicles accumulate in the liver and lungs within 5 min after systemic injection (Choi & Lee 2016). Investigators interpreted this as the RES are captured the EVs in the very early phase of circulation. After retained by the RES, EVs might be degraded. The pattern of rapid clearance is very similar to liposomes and rapidly cleared by the liver and spleen (Choi & Lee 2016). Exosome can be the best paradigm shift in RNAi therapeutics, because they are the first example of a biological, non-synthetic vehicle that allows tissue-specific, safe and

efficient siRNA delivery after systemic administration (El-Andaloussi *et al.* 2012). Even though the exosomes secreted by donor cells are non-selectively incorporated into recipient cells, the exosome uptake mechanism is different depending on the recipient cells (Horibe et al. 2018).

1.1.14 Extracellular vesicle isolation methods and challenges

A major ongoing challenge is to establish methods that will allow one to discriminate between exosomes and microvesicles and the molecular properties such as size, morphology, density, and protein composition are not yet sufficient to get a clear distinction between them (Raposo & Stoorvogel 2013). Different laboratory institutions and companies to distinguish these cargo molecules practiced different isolation methods such as size-based ultracentrifugation with sucrose or without sucrose gradient, and kits (e.g. Exoquick exosome isolation kit). Exosomes are had similar biophysical properties like virus particles and therefore, ExoQuick and Total exosome Isolation reagents contain volume-excluding polymers (e.g. polyethylene glycol, dextrans, or polyvinyls) were used (Rider et al., 2016). However, the measurements and techniques are more biased and dependent on different variables. One of the techniques was using conventional flow cytometry to distinguish the different sizes of extracellular vesicles. However, it cannot distinguish vesicles that are <300 nm in size even with novel high-resolution flow cytometry-based method except by using quantitative high throughput analysis of individuals using immunolabeled (Raposo & Stoorvogel 2013).

Isolation of extracellular vesicles in the follicular fluid of bovine was conducted by ultracentrifugation (golden standard) method as sown in (Sohel *et al.* 2013). One limitation in using differential centrifugation for isolating extracellular vesicles is co-precipitation of protein aggregates, apoptotic bodies, or nucleosomal fragments, which may lead to less sample purity and less correctly bound proteins (Momen-Heravi *et al.* 2013). To address these issues, extracellular vesicles isolated from follicular fluid, using differential centrifugation

could be separated using continuous sucrose gradient but it comes with another drawback with the low concentration of EVs.

1.1.15 Extracellular vesicle involvement during folliculogenesis and early development

The ovarian follicle, seminal fluid, endometrium, embryo, and trophoblast cells are all possible sources of EVs that have the potential to modulate maternal immune function locally (Tannetta *et al.* 2014). In recent years, much data has been collected regarding the specific components of extracellular vesicles from various cell types and body fluids using proteomic, transcriptome, and lipidomic analysis. During early development including gametogenesis, fertilization, implantation, and early embryo development are dependent on communication between the functional cells and different organs. Several studies have been performed in extracellular function in the follicular fluid of different organisms. In humans ovarian follicle, extracellular vesicles coupled miRNAs are predicted to targets that regulate the WNT, ErbB, MAPK and TGFb signaling pathways, all of which operate across the different compartments in the ovarian follicle and can contribute to follicular development, meiotic resumption and ovulation (Machtinger *et al.* 2016).

In a bovine follicular fluid study between exosomal and non-exosomal content of miRNAs in small follicles with a diameter of 4-8 mm revealed that a higher number (509) was detected in exosomal and compared to non-exosomal (356) miRNAs (Sohel *et al.* 2013). This has evidenced that, exosomal cell-to-cell communication to be the dominant way of molecule transport in the follicular environment. In the same study, exosomal miRNA expression was associated with the growth of oocyte. MicroRNAs which were found to be enriched in FF of fully grown oocytes known to be involved in ubiquitin, neurotrophin, MAPK, and insulin signaling pathways, which are linked with ovarian follicular growth, oocyte meiotic maturation and mitotic division of early embryos (Sohel *et al.* 2013). Another independent study showed that in bovine follicular fluid EVs found to carry 249 known miRNA and the

presence of 455 unknown miRNA (Navakanitworakul *et al.* 2016b). Human follicular fluid exosomal microRNAs were reported to be involved in critically important pathways for follicle growth and oocyte maturation such as WNT, MAPK, ERbB, and TGFb, that contribute to follicular development and meiotic resumption (Santonocito *et al.* 2014). Moreover, amniotic fluid stem cells derived exosomes prevent ovarian follicular atresia in chemotherapy-treated mice *via* the delivery of microRNAs in which both miR-146a and miR-10a are highly enriched and their potential target genes are critical to apoptosis (Xiao *et al.* 2016). In bovine, cumulus expansion and, and Tnfaip6 gene expression were measured following COC maturation culture. Follicular fluid EVs supported both measurable cumulus expansion and increased the expression of Ptgs2 and Ptx3 genes (Hung *et al.* 2015). In women, follicular fluid EVs reported to be enriched with miRNAs and involves in follicular growth, resumption of oocyte meiosis, steroidogenesis, and prevention of polyspermy (Machtinger *et al.* 2016).

EVs were detected in embryos cultured media and in the uterine environment and suggested to play a role during embryo-endometrium cross-talk during embryo development (Machtinger *et al.* 2016). Supplementation of outgrowth embryo-derived EVs produced during pre-implantation embryos both in vitro and in vivo had reported improving preimplantation embryonic development by increasing cell proliferation and decreasing apoptosis in blastocysts in mice (Kim et al. 2019). Conditioned media from non-manipulated human embryos cultured *in vitro* and uptake of embryo-derived EVs by human primary endometrial epithelial and stromal cells was evidenced to EVs exchange as an emerging way of communication at the maternal-fetal interface (Giacomini et al. 2017). Furthermore, the characterization of EVs during embryo culture has been reported to play an important role in inter embryo communication during bovine embryo cultured in a group (Pavani et al. 2018).

MicroRNA in endometrial epithelial cells has reported the exosome/mv-specific miRNAs to have potential targets in biological pathways highly relevant for embryo implantation. Thus, exosomes/mv containing specific miRNA are present in the microenvironment in which embryo implantation occurs and may contribute to the endometrial-embryo cross talk essential for this process (Ng *et al.* 2013). EVs have proposed to regulate physiological adaptation throughout pregnancy including the modification of maternal immune cell responses and syncytiotrophoblast function locally and systemically (Escudero *et al.* 2016). Furthermore, the co-incubation of EVs derived from bovine oviduct epithelial cell lines on the developing with bovine zygotes *in vitro* evidenced that, EVs facilitate communication between the oviduct and the embryo in the early stages of development (Lopera-Vasquez et al. 2016). Bovine oviductal EVs have been speculated to be applied to a sperm (to involve in sperm capacitation, to improve in vitro maturation rates and blastocyst yield, to improve embryo development and quality and to improve pregnancy and birth rates (Alminana and Bauersachs 2019).

Previous work on oxidative stress suggests that granulosa cells exposed to oxidative stress conditions react to stress by activating cascades of cellular antioxidant molecules which can also be released into the extracellular environment through exosomes (Saeed-Zidane et al. 2017). Different studies showed that NEB has to bring changes in the concentration of blood and follicular fluid metabolites and is associated with bad fertility. Circulating miRNAs are stable in the circulation and resistant to RNAse digestion extreme pH, high temperatures, extended storage, and multiple freeze-thaw cycles and could be used as good biomarkers (Baldassarre *et al.* 2017). Furthermore, circulatory miRNAs found to be carried by EVs in the follicular fluid and blood serum in bovine. Therefore, identifying EVs coupled with miRNAs in the follicular fluid and blood serum could help to identify non-invasive markers of metabolic stress.

1.2 Problem statement

Negative energy balance during early lactation is the main cause of fertility reduction in dairy cows. Noninvasive marker development of metabolic stress is vital for the future assisted reproductive technology in dairy cows. EVs are evolutionarily conserved communicasomes and transport various molecules including microRNAs and found in different biological fluids. Circulatory miRNAs have recently emerged as promising diagnostic biomarkers in several conditions. However, there is scarce information on the dynamic change of EV-coupled miRNAs as an indicator of metabolic stress in follicular fluid and blood serum of post-calving dairy cows. Therefore, there is a need to identify EV-coupled miRNAs in follicular fluid and blood serum of different metabolic status cows as an indicator of metabolic stress.

1.2.1 Research Hypothesis

It is hypothesized that negative energy balance influences the dynamic content of EV-coupled miRNAs in follicular fluid and blood serum in dairy cows during the postpartum period. Analysis of the global expression of EV-coupled miRNAs may help to identify specific molecules as an indicator of metabolic stress in dairy cows.

1.2.2 General objective

The main objective of the present thesis was to investigate the association of postpartum negative energy balance and the expression of extracellular coupled miRNAs in follicular fluid and blood serum of metabolically divergent cows during early post-calving.

1.2.3 Specific objectives

- To investigate the association between the dynamic change of extracellular vesicle-coupled miRNAs contents and metabolic stress in the follicular fluid and blood serum of early post-calving dairy cows
- To identify extracellular vesicle--coupled miRNAs as a predictor of the transition from negative to positive energy status in follicular fluid and blood of early post calving dairy cows
- To investigate the effect of lactation physiology on the expression of EV-coupled miRNAs in follicular fluid and blood serum
- To characterize the function of differentially expressed extracellular vesicle-coupled miRNAs in follicular fluid and blood serum of metabolically divergent cows
- To verify the uptake and effect of follicular fluid’s extracellular vesicles obtained from different metabolic status during oocyte maturation

1.3 Materials and methods

Three independent experiments were performed. The first experiment focused on screening the energy balance status of experimental cows and heifers (Fig 1.7I). The second experiment focused on the expression profiling of EV-coupled miRNAs in follicular fluid and blood serum (Fig 1.7II). The third experiment addressed the uptake of EVs after co-incubation of during cumulus-oocyte-complex. The details of the material and method are included in each of the respective chapters. Hereunder is the summary of the materials and methods are described.

1.3.1 Experimental set up of the experiment

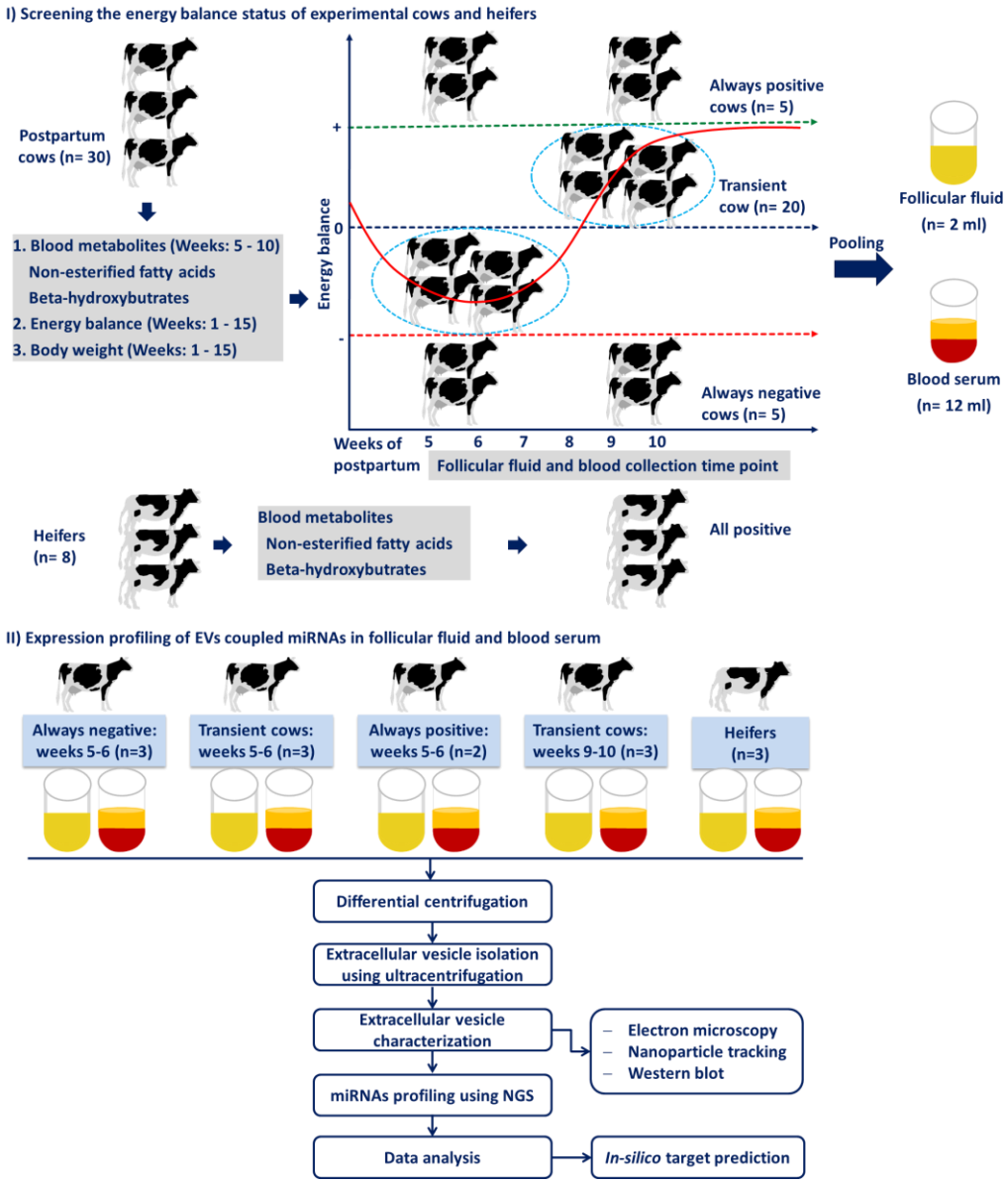

Figure 1.7: Schematic diagram showing the overall experimental design during follicular fluid and blood sample collection from cows and heifers.

1.3.2 Ethical approval of the experiment animals

Experimental animal handling was done in accordance with the 2015 German law of rules and regulations of animal protection (TierSchG & TierSchVersV). All animals were kept in the dairy farm of Frankenforst teaching and research station, University of Bonn. Experimental protocols were approved by the state office for Nature, Environment and Consumer Protection of North Rhine-Westphalia, Germany (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Deutschland) with licensing number of 84-02.04.2015.A139 for blood collection and 84-02.04.2014.A500 for Ovum Pick Up (OPU) of collecting the follicular fluid.

1.3.3 Follicular fluid and blood sample collection and handling

A total of thirty multiparous postpartum Holstein Friesian cows and eight nulliparous heifers were used for the experiment. Follicular fluid and blood samples were collected from all cows at week; 5, 6, 7, 8, 9 and 10 of the postpartum period. Follicular fluid samples were collected from large follicles with a diameter of >10 mm using the Ovum Pick Up technique. Briefly, puncturing of the ovary's follicle was performed using an ultrasound-guided instrument to recover the follicular fluid. Therefore, follicular fluid samples obtained from each cow at each time point were centrifuged individually at 500 g for 5 minutes to separate the follicular fluid from the granulosa cells and stored at -80 °C for the next step. In parallel, blood samples were collected from all postpartum cows and heifers at each OPU time point. A total of 20 ml of blood samples were used for serum separating using a serum separation tube (Sarstedt AG & Co. KG, Nümbrecht, Germany). In addition to postpartum cows, follicular fluid and blood samples were collected from nulliparous heifers using a similar procedure for five OPU sessions. Blood samples were centrifuged at the time of collection at 2500 g for 10 minutes to separate the serum from the clotting factors and the blood serum samples were stored until the next step.

1.3.4 Analysis of blood serum metabolites

Blood serum metabolites analysis was performed for both postpartum cows and heifers. Analysis of two metabolites; non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (β-OHB), was performed using commercial kits as previously published (Frieten *et al.* 2017). NEFA concentration was quantified using series NEFA-HR (Wako Chemicals GmbH, Neuss, Germany). The concentration of β-OHB was quantified using the Ranbut D-3-hydroxybutrate using BHBA (# RB1008) kit (Randox laboratory Limited, United Kingdom) as previously described in (Frieten *et al.* 2017).

1.3.5 Body weight measurement and overall energy balance analysis

The body weight of each cow was measured daily for the first 15 weeks postpartum. Therefore, body weight curves were generated in weakly based by taking the mean and the standard deviation. The graphs represented the mean ± standard deviation of all cows were generated in GraphPad prism 5. Furthermore, an individual cow's overall energy balance was performed. For this individual cow' dry matter intake and milk production were measured in daily based. Afterward, the overall energy balance of individual cows was calculated using the following equation:

Energy balance (EB) (MJ NEL/d) = Energy intake (EI) - Energy consumption (EC)

Where Energy intake (EI): Dry matter intake (MJ) + Concentrate intake (MJ)

Energy consumption (EC): Maintenance energy (MJ) + milk energy (MJ)

1.3.6 Follicular fluid and blood serum sample pooling

Based on these three criteria, for metabolic status calculation, animals were grouped into three categories namely: Transient cows (TCs) (n=20), Always Negative cows (ANCs) (n=5) and Always positive cows (APCs) (n=5) (Fig. 2). Follicular fluid samples from cystic ovaries and blood contamination are discarded from the analysis. Follicular fluid samples from TCs at

weeks 5 and 6 (n=11) and at weeks 9 and 10 (n=7) were separately pooled to create three biological replicates. Follicular fluid collected from ANCs (n=2) and APCs (n=2) at weeks 5 and 6 were pooled to generate two biological replicates per group of cows.

1.3.7 Isolation of extracellular vesicles from follicular fluid and blood serum

Following pooling, EVs were isolated using differential centrifugation followed by the ultracentrifugation protocol. After the removal of cell debris, samples were diluted with an equal amount of PBS to reduce the viscosity. Follicular fluid or blood serum samples were centrifuged at 500 g to remove cell debris followed by another centrifugation at 6000 g to remove the apoptotic body. The supernatant was transferred to a new tube and large vesicles were removed by centrifugation at 18,000 xg for 30 min. Afterward, the supernatant containing EVs was transferred to a new polypropylene ultracentrifugation tube and centrifuged at 120,000 -130,000 xg for 60 min to pellet the extracellular vesicle pellets. Extracellular vesicles' pellets were suspended in 5 ml of PBS and washed three times at 120,000 xg. After washing steps, EVs pellets were resuspended in PBS and stored in -80 ^{O}C for further characterization and molecular analysis.

1.3.8 Nanoparticle tracking and electron microscopy analysis of EVs

Nanoparticle tracking analysis of EVs was performed to determine their concentration and the size distribution using Nano Sight NS300 (Malvern Instruments, Malvern, UK) following manufacturer protocols. Briefly, 25 µl suspended in EVs solution was diluted 1 ml of PBS and used for five video recordings. The recorded data were analyzed using NTA version 3.2. Furthermore, EVs morphology was characterized using a transmission electron microscope (Ziess EM109, Carl Zeiss). Briefly, 20 µl drops of purified suspended EVs were poured to parafilm and covered by Formvar-carbon-coated grids and then incubated with 2 % uranyl acetate. Grids containing EVs were washed with drops of PBS and examined under an electron microscope.

1.3.9 Western blotting

Western blotting was performed to detect the protein marker of EVs recovered from follicular fluid and blood samples. For this, EVs protein was extracted using radioimmunoprecipitation assay buffer (RIPA) (Sigma-Aldrich, Germany). Extracted protein (30 µg) was loaded on an SDS gradient gel, electrophoresed, and blotted to nitrocellulose membrane using Biorad Pac 3000 transblot (BioRad, USA). Primary antibodies of Alix and CD63 (System biosciences, Palo Alto, Canada) were diluted with the blocking solution in a ratio of 1:300. Afterward, membranes were incubated with Goat Anti-Rabbit secondary antibody (System biosciences, Palo Alto, Canada) for 1 hr. Protein bands were visualized using the ChemiDoc™ XRS system (Bio-Rad Laboratories GmbH, USA).

1.3.10 Isolation of Total RNAs enriched with miRNAs

Total RNA enriched with miRNAs was isolated from the EVs using the exosomal RNA isolation kit (Norgen, Canada) according to the manufacturer's protocol. Afterward, the quality of total RNA including miRNAs was assessed using NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Germany) and Agilent 2100 Bioanalyzer (Agilent Technologies, Canada).

1.3.11 Library preparation and Next-Generation sequencing

Illumina based sequencing platform library preparation was done using QIAseq miRNA Library Prep kit using NextSeq500 according to the manufacturer protocol (Qiagen, Heliden, Germany). Briefly, each sample of total RNAs of 160 ngs was used for the QIAseq miRNA workflow. A total of 5 µl total RNAs were converted into microRNA NGS libraries. Adapters containing UMIs were ligated into RNAs samples and converted into cDNA. Afterward, the cDNA was amplified using PCR (22 cycles). Followed the PCR, samples were purified for further procedure. Library preparation quality control was performed using Bioanalyzer 2100 (Agilent) and TapeStation 4200 (Agilent). Based on the quality of the inserts and

concentration measurements, libraries were pooled in equimolar ratios. Library pools were quantified by the qPCR ExiSEQ LNA™ Quant kit (Exiqon). Sequencing was performed on a NextSeq500 sequencing instrument according to the manufacturer's instructions. Raw data were demultiplexed and FASTQ files for each sample were generated using the bcl2fastq software (Illumina Inc.).

1.3.12 Data analysis

The quality of raw FASTQ data was assessed using FastQC version 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Afterward, data analysis was performed according to the XploreRNA pipeline (Qiagen, Germany). Briefly, adaptors were trimmed from the raw sequences using Cut adapt (1.11) and mapped against the indexed bovine reference genome (UMD3.1) using Bowtie2 (2.2.2) software with a criterion of one mismatch in the first 32 bases of the reads without insertion or deletion. Sequence reads, which were aligned to the bovine reference genome, were then used for annotation against bovine precursor miRNAs and mature miRNAs in the miRbase database and release 20. Differential expression analysis was performed on TMM normalized expression values using the EdgeR statistical software package (Bioconductor, http://www.bioconductor.org/). A miRNA with a $\log_2$ fold change of $1 \geq \log2 \leq -1$ and a p-value < 0.05 was considered as statistically significant. In addition to that, a false discovery rate of 0.25 for the follicular fluid samples and 0.3 for the blood serum samples were used as a threshold. The target prediction of differentially expressed miRNAs was analyzed using different bioinformatics tool including DIANA-miRPath v3. Moreover, miRNA-gene network analysis was performed using ingenuity pathway analysis and Network analyst (Xia *et al.* 2015).

1.3.13 Co-incubation of extracellular vesicles during oocyte maturation

EVs recovered from follicular fluid were labeled and co-incubated with immature cumulus-oocyte-complex (COC) for 22 hrs during the *in* vitro maturation. Therefore, EVs recovered

from transient cows at week 5-6, and at week 9-10, always negative at week 5-6, always positive at week 5-6 and heifers. EVs were labeled using green dye PKH67 Fluorescent Cell Linker Kits labeling kit according to the manufacturer protocols (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's protocol prior to co-incubation. Briefly, prior to co-incubation, suspended EVs (25µl) in PBS were mixed with diluted 125µl diluent ion C. The EVs mixture and Dye mixture were incubate for 5 min. Afterward, the labeling was stopped by adding an equal volume of extracellular vesicles free Foetal Bovine Serum for 1 min to allow binding of excess dye and centrifuged by ultracentrifugation for 30 min at 100000x g at 4°C. After labeling, EVs were co-incubated with immature COC for 22 hrs. Matured COC were fixed in 4% paraformaldehyde. Imaging of COC EVs uptake was done after 22 hrs of maturation under confocal microscopy. After fixation, COCs were stained with Hoechst 33342 stain (Sigma, Munich; Germany). COCs were mounted in mounting medium containing DAPI on a slid and visualized under the CLSM LSM-780 confocal laser-scanning microscope (Zeiss, Germany).

1.4 Results

In this chapter, the main findings of this PhD dissertation are highlighted. The detailed results figures, tables, and their legends are presented in chapter-2 and 3 of this thesis.

1.4.1 Quantification of blood serum metabolites revealed three specific types of cows

To investigate the clinical symptoms of NEB, the level of ketone bodies in the blood serum for all experimental animals were measured. Based on the blood serum analysis of NEFA and BOH analysis, 67% (20 out of 30) of the postpartum cows were a transient cow. Those types of cows were having a high level of NEFA ≈ 0.82±0.32 mmol/L and BHO ≈0.85±0.36 mmol/L at week 5 of postpartum and were able to start recovering from week 7-8 and fully recovered at week 10 with reduced metabolite level. Out of the remains, 17% (5 out of 30) of the cows were having a high level of blood serum in a range of NEFA ≈0.69±0.08 to

1.05±0.06 mmol/L and BHO ranges of ≈0.78± 0.17 to 0.98±0.02 mmol/L through the study period and considered as always negative cows (ANCs). The third types of cows were always positive cows representing 17% of the experimental animals (5 out of 30). These cows were having a lower concentration of NEFA ≈0.26±0.19 - 0.40±0.10 mmol/L during the study period and ß-hydroxybutyrate concentration of 0.42 ± 0.17± 0.53 ±0.15 mmol/L during the study period. The blood serum concentration of those deleterious metabolites was as low as 0.07± 0.03 to 0.19±0.13 mmol/L of NEFA and 0.38±0.14-0.46±0.05 mmol/L of BHO for experimental heifers.

1.4.2 A variation on body weight and energy balance status of postpartum cows

The results of the body weight curve assessment of individual cows for consecutive 15 weeks of post-calving revealed that the majority of the cows (20 out 30) were found to lose their body weight as early as 2nd week of postpartum and start to recover after weeks 7 and 8. These groups of cows were considered as transient cows (early loss and late gain). The rest of the cows (10 out of 30) were found to either loss or gain their body weight continuously throughout the study period. This supports the continues onset of NEB in some portion of cows in case of ‘always negative cow’ or without any onset of NEB at all in case of ‘Always positive cows’. Furthermore, the overall energy balance calculation on the energy status of pattern postpartum cows based on the dry matter intake was in support of the blood metabolites results and body weight curves of the postpartum experimental cows.

1.4.3 Characterization of EVs recovered from follicular fluid and blood serum

Characterization of the EVs using the nanoparticle tracking analysis revealed that follicular fluid and blood serum samples are enriched with a huge number of extracellular vesicles. The size of EVs ranged from 124.8 -147.1nm in follicular fluid and 113-141 nm in diameter in blood serum. This was supported by electron microscope analysis morphology characterization and the EVs have acceptable morphology and shape. The concentration of

particles recovered from follicular fluid was between 4.43e+008 ± 1.13e+008 and 2.39e+009±5.01e+007 particles/ml. The specificity of EVs isolated from follicular fluid or blood serum from each experimental group was evidenced by the detection of EV protein markers, namely CD63 and Alix, using protein-specific antibodies. Extracellular vesicle´s RNA quality assessment showed the absence of 18s and 28s bands, which indicate the absence of cellular RNA contamination.

1.4.4 EVs in follicular fluid and blood serum are enriched with small RNAs

Next-generation sequencing analysis of EV-coupled miRNAs revealed high-quality raw data with an average of 6.7 million reads in follicular fluid and 37.7% average genome-mapping rate. For the blood serum, NGS revealed 12.2 million reads per sample with an average genome-mapping rate of 16.8%. Global approach detection of EV-coupled miRNA revealed that, 356 known miRNAs and 156 putative novel miRNAs across all follicular fluid samples. Out of those, 255 of them were detected in all groups. Interestingly, several miRNAs were uniquely detected in specific experimental groups as the metabolic status of the cows changed and the highest numbers of unique miRNAs were detected in APCs (18 miRNAs). Moreover, we have detected eight unique miRNAs in ANCs, seven in TCs, and four unique miRNAs heifers, respectively. In blood serum, a total of 179 known and 118 novel EV-coupled miRNAs have detected across the different groups of cows.

1.4.5 Negative energy balance suppressed the release of EVs coupled miRNAs in follicular fluid and blood serum

Differential expression analysis of miRNAs recovered from the follicular fluid of cows in different metabolic status revealed that cows with different metabolic statuses exhibited different miRNA expression profiles. Pairwise comparisons among the APCs, ANCs, TCs to their control heifers showed that the differentially expressed (DE) miRNA profiles of TCs and APCs were relatively similar whereas ANCs exhibited a more distinct miRNA profile Most of

the differentially expressed miRNAs were downregulated in ANCs, while a large proportion of the TCs and APCs DE miRNAs exhibited induced expression compared to control heifers. Furthermore, the differential expressed analysis of miRNAs between the ANCs compared to APCs revealed the downregulation of all five differentially expressed miRNAs (bta-miR-2285, bta-miR-451, bta-miR-132, bta-miR-486, and bta-miR-874).

The target prediction of those downregulated miRNAs revealed their involvement in various pathways associated with metabolic processes and oocyte follicular growth, including insulin signaling, estrogen signaling, vitamin B6 metabolism, fatty acid biosynthesis, fatty acid metabolism, fatty acid elongation, progesterone-mediated oocyte maturation, and others. Moreover, DE expression profiling of EV-coupled miRNAs in ANCs to metabolically unstressed heifers revealed major downregulation and out of 37 miRNAs, 25 of those were downregulated (including bta-miR-20b, bta-miR-363, bta-miR-132, bta-miR-184, bta-miR-451, bta-miR-223, bta-miR-18a, bta-miR-122, bta-miR-147, bta-miR-21-3p, bta-miR-150, bta-miR-146a, bta-miR-138, bta-miR-193b, bta-miR-155) in APCs or lactating cows and only 12 miRNAs were upregulated in follicular fluid.

The hierarchical clustering of EV-coupled miRNAs in all biological replicates of different metabolic status cows in blood serum showed that major downregulation of EV-coupled miRNAs in ANCs and TCs whereas the opposite was observed in APCs and heifers replicates. Analyzing the effect of NEB on EV-coupled miRNAs in the blood serum revealed major downregulation of the in the metabolically stressed cows in blood serum. Differential expression analysis between TCs compared to the heifers has revealed a downregulated of 28 and upregulated 13 miRNAs. Furthermore, the ANCs compared to APCs reveals differential expression of five miRNA in which downregulation of bta-miR-145 and miR-375 and upregulation of bta-miR-133a, miR-9-5p, and miR-184 in blood serum of ANCs. Furthermore, the comparison between ANCs at weeks 5 & 6 and heifer has revealed a

downregulation of 18 out of 29 miRNAs. In order to observe the trend of miRNAs expression during the shortage of energy, we have performed a pairwise compared of the similarities and differences on EV-coupled miRNAs abundance between the ANCs vs heifers and in the TCs at their week 5 & 6 vs heifers. Interestingly, we have found that 17 miRNAs to be downregulated in both comparisons and three miRNAs were upregulated in blood serum.

1.4.6 EVs coupled miRNAs as predictors of transition from negative energy to positive energy status during early lactation

To identify specific miRNAs as an indicator of recovery, a comparison of the ANCs and TCs has performed in both follicular fluid and blood serum samples. Follicular fluid EV-coupled miRNA profiles of ANCs and TCs at weeks 5 & 6 were compared to determine candidate miRNAs that may be indicators for the transition to positive energy balance in the later stages of lactation (weeks 9 and 10). In this comparison, miRNAs (i.e. bta-miR-34b, bta-miR-34c, bta-miR-449a, and bta-miR-132) were downregulated in ANCs compared to TCs, which have recovered from their metabolic stress at weeks 9 & 10. Comparison of differentially expressed miRNAs between EVs recovered from follicular fluid collected from transient cows at weeks 5-6 and weeks 9-10 revealed that bta-miR-34b, bta-miR-34c, and bta-miR-449a to be upregulated, whereas two miRNAs, bta-miR-451 and bta-miR-592, were downregulated at weeks 5-6.

Furthermore, analysis of EV- coupled miRNAs in blood serum as an indicator by comparing the ANCs vs TCs at weeks 5 and 6 showed that upregulation of all nine differentially expressed miRNAs (bta-miR-1, bta-miR-122, bta-miR-133a, bta-miR-184, bta-miR-192, bta-miR-206, bta-miR-215, bta-miR-375, and bta-miR-9-5p). Furthermore, the comparison of TCs at week 5-6 compared to their week 9-10 revealed the downregulation of all differentially expressed miRNAs revealed that the downregulation of ten miRNAs including bta-miR-122,

bta-miR-133a, bta-miR-192, bta-miR-206, bta-miR-215, bta-miR-375 and bta-miR-9-5p which were upregulated in the comparison of ANCs vs TCs.

1.4.7 Conserved EV-coupled miRNAs expression in follicular fluid and blood serum

The expression analysis of EV-coupled miRNA in post-calving cow's follicular fluid and blood serum as an indicator of follicular fluid microenvironment during NEB has performed. For this pairwise comparison of EV-coupled miRNAs in ANCs and heifers in both samples showed that four miRNAs were significantly expressed and with a similar pattern. Out of those, bta-miRNA-122 and bta-miRNAs-150 were downregulated whereas the other two; bta-miRNA-9-5p and bta-miRNA-196 were upregulated in both comparisons.

1.4.8 Lactation induced the changes in EVs coupled miRNAs in follicular fluid

Investigation of lactation induced EV-coupled miRNAs in both follicular fluid and blood serum have done by comparing the APCs and heifers. The result revealed that, compared to heifers out of 38 differentially expressed miRNAs, 31 miRNAs were upregulated in APCs or lactating cows compared to heifers in follicular fluid (including bta-miR-135a, bta-miR-2285z, bta-miR-502a, bta-miR-449a, bta-miR-874, bta-miR-1291, bta-miR-1246, bta-miR-320b, bta-miR-130a, bta-miR-331-5p, bta-miR-23b-3p, bta-miR-107, bta-miR-362-3p, bta-miR-873, bta-miR-320a). Unlike the follicular fluid, lactation does not induce EVs- coupled miRNAs in blood serum and only EV-coupled miRNAs; bta-miR-206 and bta-miR-133a have found differentially expressed in APCs compared to heifers.

1.4.9 EVs recovered from the follicular fluid can be up-taken by cumulus cells but not by the oocytes

Co-incubation of extracellular vesicles recovered from follicular fluid with cumulus-oocyte-complexes showed uptake of extracellular vesicles by the cumulus cells but not by the oocyte. This result was accompanied by a lower tendency of oocyte maturation rate in oocytes co-

incubated with extracellular vesicles recovered from follicular fluid from cows at negative energy balance compared to heifers.

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Chapter 2: Extracellular vesicle-coupled miRNA profiles in follicular fluid of cows with divergent post-calving metabolic status

Extracellular vesicle-coupled miRNA profiles in follicular fluid of cows with divergent post-calving metabolic status

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2.1 Abstract

Most high-yielding dairy cows enter a state of negative energy balance (NEB) during early lactation. This, in turn, results in changes in the level of various metabolites in the blood and follicular fluid microenvironment which contributes to disturbed fertility. Extracellular vesicles (EVs) are evolutionarily conserved communicasomes that transport cargo of miRNA, proteins, and lipids. EV-coupled miRNAs have been reported in follicular fluid. However, the association between postpartum NEB and EV-coupled miRNA signatures in follicular fluid is not yet known. Energy balance analysis in lactating cows shortly after post-calving revealed that the majority of the cows exhibited transiently negative energy balance levels, whereas the remaining cows exhibited either consistently negative or consistently positive energy levels. Metabolic status was associated with EV-coupled miRNA composition in the follicular fluid. Cows experiencing NEB showed reduced expression of a large number of miRNAs while cows with positive energy balances primarily exhibited elevated expression of EV-coupled miRNAs. The miRNAs that were suppressed under NEB were found to be involved in various metabolic pathways. This is the first study to reveal the presence of an association between EV-coupled miRNA in follicular fluid and metabolic stress in dairy cows. The involvement of differentially expressed miRNAs in various pathways associated with follicular growth and oocyte maturation suggests the potential involvement of specific follicular miRNAs in oocyte developmental competence, which may partially explain reduced fertility in cows due to post-calving metabolic stress.

2.2 Introduction

Selection for milk yield in dairy cows in the last 70 years has resulted in a significant increase in milk yield and concomitant reduction in fertility. In modern dairy cows, heifers have calving rates of ~ 55 – 60% that reduces to ~ 35 - 40% in lactating cows [1]. One of the main causes of reduced fertility rates is postpartum negative energy balance (NEB) during early

lactation, a condition in which a cow's energy demand for maintenance and lactation is higher than its dietary energy intake. Negative energy balance has been documented to have the strongest association with declining fertility [2–5]. During NEB, cows have a shortage of circulating glucose and their body starts to metabolize fat reserves through lipogenesis and/ ketogenesis. This leads to an increase of undesired substances in the blood such as non-esterified fatty acids (NEFA) and beta-hydroxybutyrate, which are known to have a negative effect on folliculogenesis [6,7]. This disturbance in blood metabolite levels is then directly reflected in the follicular fluid microenvironment [8]. As a consequence, in the first few months of the post-calving period, ovarian follicle development occurs in a compromised endogenous metabolic microenvironment [9] and results in significantly less viable preantral follicles [10].

Follicular fluid (FF) provides a nurturing microenvironment for the development of oocytes by allowing them access to various nutrients and hormones that are produced from surrounding somatic cells. Follicular fluid is a product of both blood plasma constituents that cross the blood follicular barrier and secretions from granulosa and theca cells [11]. Studies utilizing gas chromatography mass spectrometry to assess FF metabolite levels have reported higher concentrations of NEFA and b-hydroxybutyrate and lower concentrations of glucose, insulin, and insulin-like growth factor in lactating cows compared to non-lactating cows and heifers [12]. In addition, supplementation of NEFA during *in vitro* oocyte maturation had a negative effect on maturation, fertilization, cleavage, blastocyst rates, and the number of late apoptotic cumulus cells [13]. Moreover, environmental factors like heat stress are also known to aggravate the consequences of NEB in high yielding dairy cows by altering biochemical concentrations in the follicular fluid of dominant follicles with profound effects on oocyte and granulosa cell quality [14]. Since oocyte quality is known to determine the number of transferable blastocysts, identification of the best quality oocytes prior to IVF has been the main focus in the field of assisted reproductive technology [11,15]. As such, biochemical

reactions like brilliant cresyl blue staining [15] and morphological parameters are utilized to select oocytes invasively. Therefore, molecules such as DNA, mRNA, miRNAs, lipids, and proteins that are released into follicular fluid from surrounding follicular cells via extracellular vesicles during the cell-to-cell communication could serve as non-invasive molecular markers for oocyte competence.

Extracellular vesicles (EVs) are evolutionarily conserved nano-sized cargo-carrying molecules released from both prokaryotic and eukaryotic cells to deliver signals to target cells [16,17]. Extracellular vesicles have been detected in various biological fluids including nasal mucosal fluid [18], cerebrospinal fluid [19], breast milk [20], saliva [21], umbilical cord blood [22], urine [23,24], amniotic fluid [24], bovine follicular fluid [25,26] and semen [27]. Extracellular vesicle is a general term encompassing several different vesicle types, including exosomes, microvesicles, apoptotic vesicles, and, in pathological situations, necrotic debris. They can be released by cells constitutively or in response to specific stimuli or cell stressors [28]. Most studies have primarily concentrated on the content of small nano-sized vesicles: exosomes and ectosomes. Exosomes are formed from internalized endocytic vesicles and are constitutively secreted from the cell [28] whereas ectosomes are ubiquitous vesicles assembled at and released from the plasma membrane [29]. Extracellular vesicles play a vital role in cell-to-cell communication and carry a huge number of proteins, lipids, mRNA, and microRNAs (miRNAs) that can be delivered to and function in other cells [17]. Thousands of exosome-mediated molecules have been documented, including about 9,769 Proteins, 3,408 mRNAs, 2,838 miRNAs, and 1,116 lipids [30]. In the early stages of the human reproductive process, the ovarian follicle, seminal fluid, endometrium, embryo, and trophoblast cells are all possible sources of EVs that have the potential to locally modulate maternal immune function [28]. Extracellular vesicle-mediated miRNAs have been detected in the follicular fluid of bovine[25], human [31], and other species. We and others previously determined that exosomal miRNAs in

bovine and human follicular fluid are associated with developmental potential of oocytes [25,31]. Due to extensive cell-to-cell communication in the follicular environment, we postulate that metabolic stress can greatly affects EV-mediated release of molecules and subsequently follicular growth and oocyte maturation. Therefore, the aim of this study was to investigate the association between the expression of EV-coupled miRNAs in bovine follicular fluid and postpartum metabolic stress in Holstein-Friesian cows by comparing metabolically stressed vs non-stressed cows. Furthermore, we aimed to investigate the effect of lactation physiology on the expression of EV-coupled miRNAs in follicular fluid by comparing metabolically non-stressed lactating cows versus heifers.

2.3 Results

2.3.1 Quantification of blood serum metabolites

In the present study, the energy status of experimental cows and heifers was assessed using blood metabolite analysis. Non-esterified fatty acids (NEFA) and Beta-hydroxybutyrate (β-OHB) were profiled at different time points post-calving. As shown in Fig. 2.1a, average NEFA concentration for experimental cows decreased from 0.73 ± 0.0.31mmol /L at week 5 to 0.47 ± 0.032mmol/ L at week 10. Similarly, the concentration of ß-OHB decreased from 0.78 ± 0.33mmol/L at week 5 to 0.57 ± 0.21mmol/L at week 10 as shown in Fig. 2.1b. The average NEFA and ß-OHB concentrations in heifers were ≤ 0.2mmol/L and ≤ 0.5mmol/L, respectively, as shown in Fig. 2.1c and d. Moreover, individual variations were observed among the experimental cows and 66% (20/30) transiently exhibited high concentrations of NEFA and ß-OHB, whereas 16% of the cows consistently exhibited high concentration of both metabolites at all time-points of the analysis. Interestingly, 16% of the cows exhibited lower concentrations of NEFA and ß-OHB through-out the early lactation period (weeks 5-10). Cows were considered to be in negative energy balance when NEFA and ß-OHB

concentrations in serum were > 0.55 mmol/L and > 0.65 mmol/L, respectively, as applied previously [32, 33].

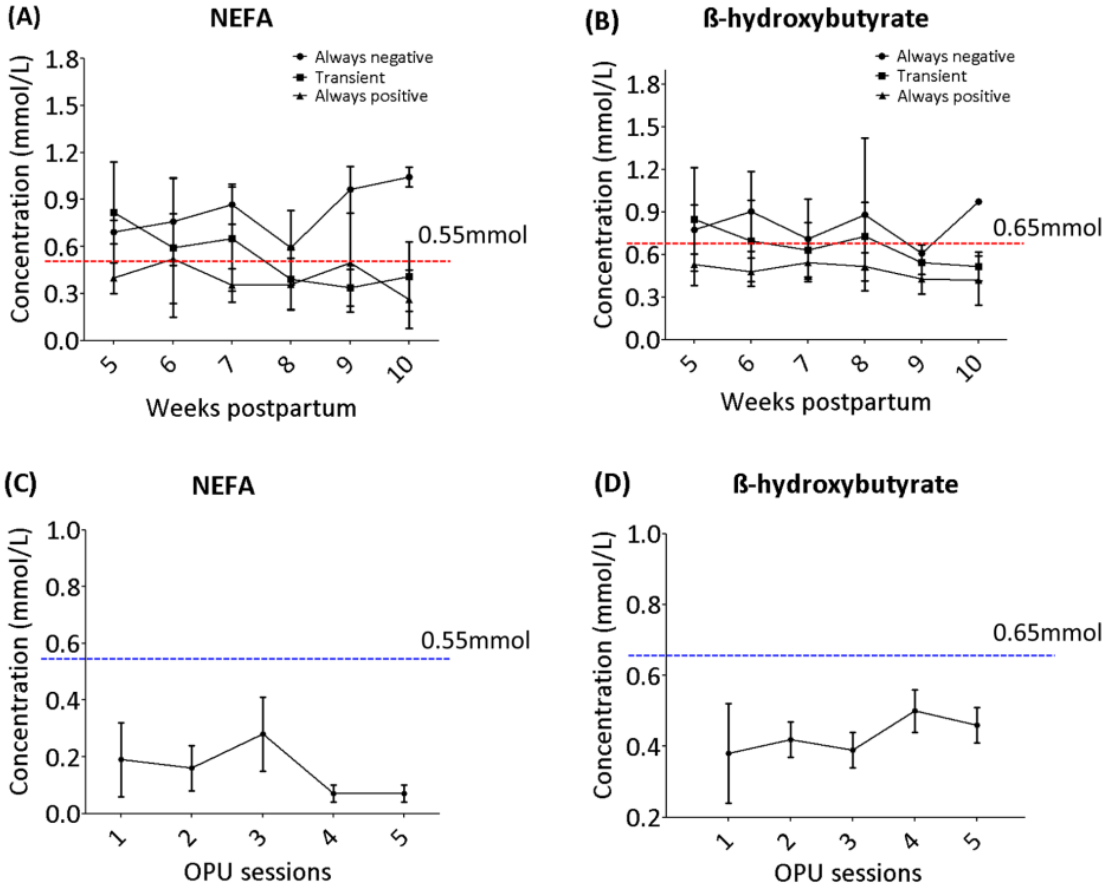

Figure 2.1: Blood serum metabolite profiles of postpartum cows and heifers **a)** The average weekly based concentration of NEFA for different metabolic status of population of postpartum cows early post-calving (weeks 5-10); **b)** The average weekly based concentration of ß-hydroxybutyrate for different metabolic status of postpartum cows (weeks 5-10); **c)** The average concentration of NEFAs per OPU session for control heifers; **d)** The average concentration of ß-hydroxybutyrate per OPU session for heifers. The dashed lines in all graphs indicate the threshold level of metabolites to categorize animals as having a negative or positive energy balance. **Always negative cows:** cows that have a high concentration of NEFA and ß-hydroxybutyrate throughout the experimental period weeks 5-

10. **Transient cows**: cows that have high concentration of NEFA and ß-hydroxybutyrate at week 5-6 and reduce to very low level starting from weeks 7-8 until week 10. **Always positive cows:** cows that have low concentration of NEFA and ß-hydroxybutyrate throughout the experimental period week 5-10.

In addition to metabolic profiles, cows' body weight measurements were performed during the experimental period and body weight curves were generated as indicated in supplementary Fig. S1. Moreover, measurement of energy status of individual cows was performed based on dry matter intake and energy expenditure for milk yield and maintenance. Based on their feed intake and milk yield the energy status of individual cows was calculated as follows. Energy balance (EB) (MJ NEL/d) = Energy intake (EI) - Energy consumption (EC). Individual cow energy balances are available as supplementary Fig. S2. Based on the combination of metabolic profiles, body weight curves, and energy balances between weeks 5 and 10 post-calving, all experimental cows were categorized into three groups: 1) Always negative cows (ANCs): cows that showed negative energy balance at all times between weeks 5 and 10; 2) Always positive cows (APCs): cows that showed positive energy balance at all times between weeks 5 and 10; 3) Transient cows (TCs): cows which showed negative energy balances at weeks 5-6 and recovered at weeks 9-10 by showing positive energy balance. While 20 out of 30 (66%) of the postpartum cows were 'transient cows' (TCs), each ANC and APCs group represents 17% (five out of 30). The distribution of the experimental animals based on the energy status is illustrated in Fig. 2.2.

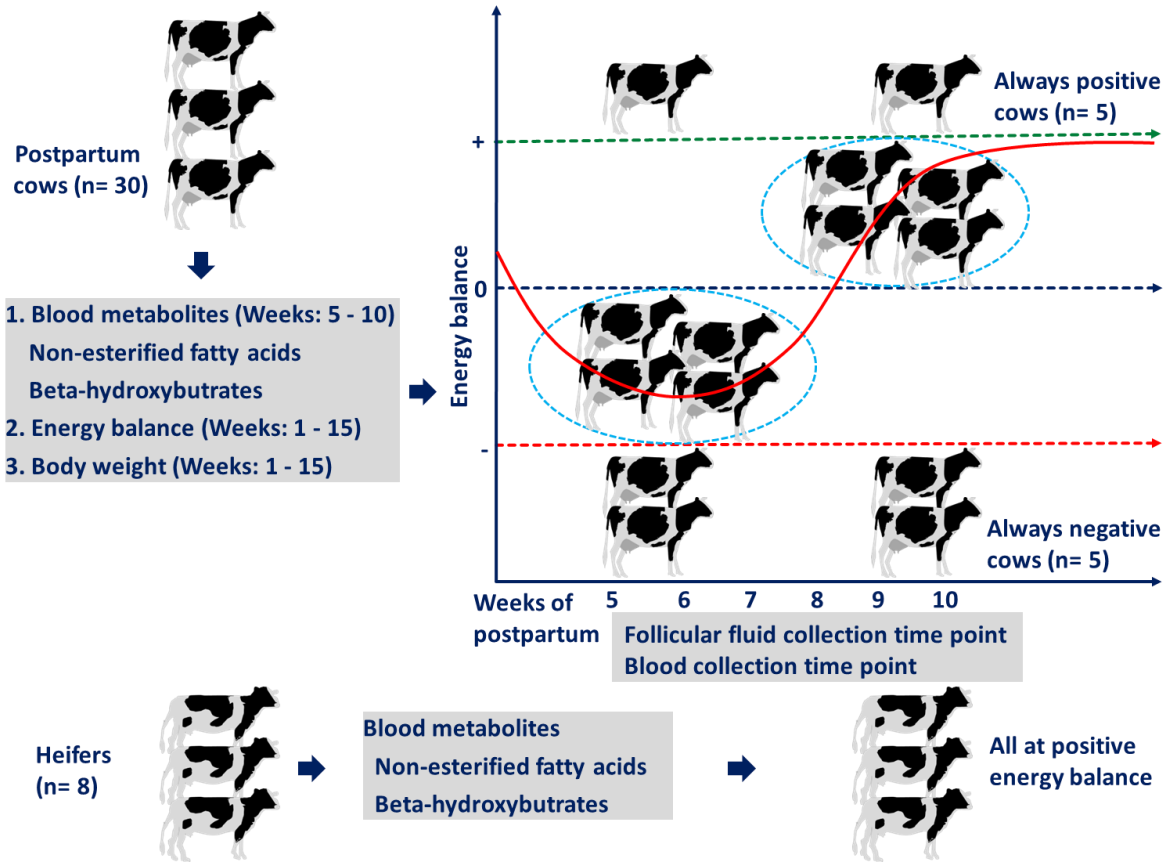

Figure 2.2: Summary of different categories of cows based on energy status as determined from blood serum metabolites, energy balance, and body weight curve of individual animals. Blood serum metabolites were measured weekly weeks 5 to 10. Individual cow body weight was measured daily (supplementary Fig. 2.S1). The overall energy balance of individual cows was measured based on their dry matter intake on a daily basis (supplementary Fig-2.S2).

2.3.2 Characterization of extracellular vesicles and isolated RNAs

Following isolation of EVs by ultra-centrifugation, morphological characterization of EVs was done using transmission electron microscope as shown in Fig. 2.3a. Furthermore, the concentration and the size distribution of extracellular vesicles isolated from follicular fluid of cows with different statuses were quantified using a nanoparticle tracking system. The size of follicular fluid EVs was found to be within the range of 124.8 ± 2.4 to 147.1 ± 5.6nm in

diameter as shown in Fig. 2.3b. The concentration of particles recovered from follicular fluid was between 4.43e+008 ± 1.13e+008 and 2.39e+009 ±5.01e+007 particles/ml. The specificity of EVs isolated from follicular fluid from each experimental group was evidenced by the detection of EV protein markers, namely CD63 and Alix, using protein-specific antibodies as indicated in Fig. 2.3c. Extracellular vesicle´s RNA quality assessment showed the absence of 18s and 28s bands, which indicate the absence of cellular RNA contamination (Fig. 2.3d).

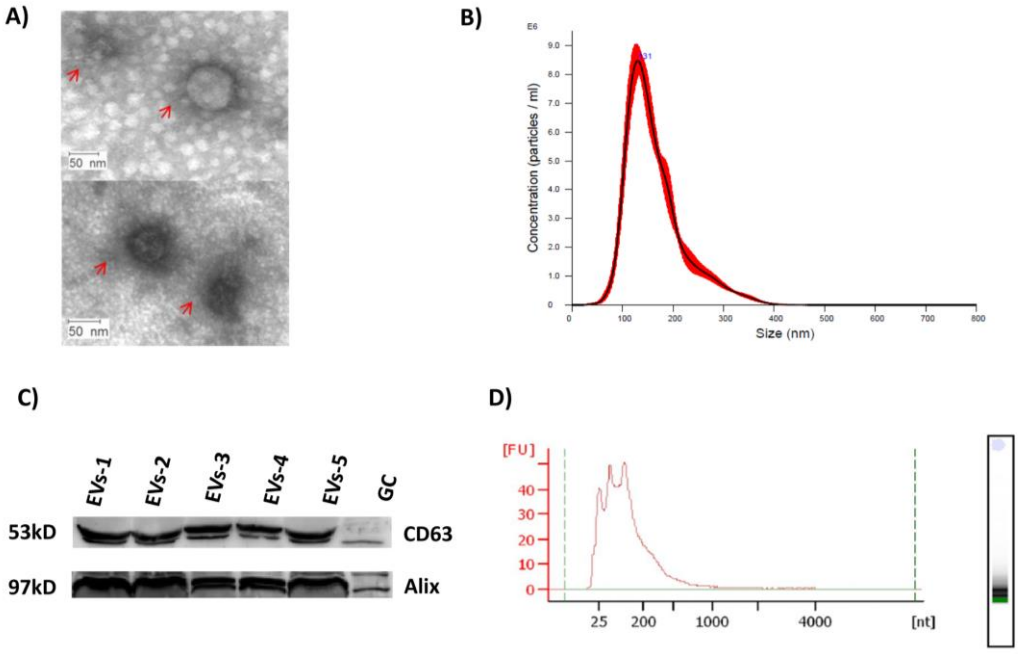

Figure 2.3: Morphological and molecular characterization of EVs recovered from follicular fluid; A) Electron microscope image of EVs. The red arrow shows the EVs morphology. b) A representative nanoparticle tracking analysis of EVs. c) Detection of EV protein markers. The numbers represent different sources EVs 1: EVs of TCs (weeks 5 - 6), 2: EVs of TCs (weeks 9 - 10), 3: EVs of ANCs, 4: EVs of APCs, 5: EVs of heifers and GS: Granulosa cells and the full image indicated in supplementary fig. 2.S3. d) A representative electropherogram image of EVs RNA quality analysis using Agilent bioanalyser.

2.3.3 Sequence quality and mapping of RNAseq data from different small RNA samples

Next-generation sequencing was performed using the Illumina-based NextSeq500 instrument with an average number of 12 Million reads per sample for which 75 nucleotide single-end reads were retained. Quality control of the sequenced data was performed before and after mapping for all biological replicates. Sequenced raw data for all replicates had a Q-score above 30 (< 1 incorrect base call 1 every 1000 bases). Following this, mapping of the sequencing data was performed against the reference sequence. Sequencing of EV-coupled small RNAs generated an average 6.7 million reads per sample and the average genome-mapping rate was 37.7%. The raw sequencing reads and the processed data have been deposited in NCBI's Gene Expression Omnibus with GEO accession number GSE129367.

2.3.4 Global detection of EVs mediated miRNAs and small RNAs in follicular fluid of different metabolic status cows

Sequencing results showed that a total of 356 known miRNAs were detected across all analyzed samples, 255 of which were commonly detected in all experimental groups as shown in Fig. 2.4. However, some miRNAs were uniquely detected in specific experimental groups and the highest numbers of unique miRNAs were detected in APC samples. APC samples had 18 unique miRNAs, including bta-miR-2400, bta-miR-181c, bta-miR-2284k, bta-miR-2285n, bta-miR-2285j, bta-miR-2284ac, bta-miR-2285v, bta-miR-330, bta-miR-2436-5p, bta-miR-2284v, bta-miR-33a, bta-miR-124b, bta-miR-2382-3p, bta-miR-135a, bta-miR-2285ab, bta-miR-665, bta-miR-153 and bta-miR-2426. Moreover, eight, seven, and four unique miRNAs were detected in ANCs, TCs, and heifers, respectively.

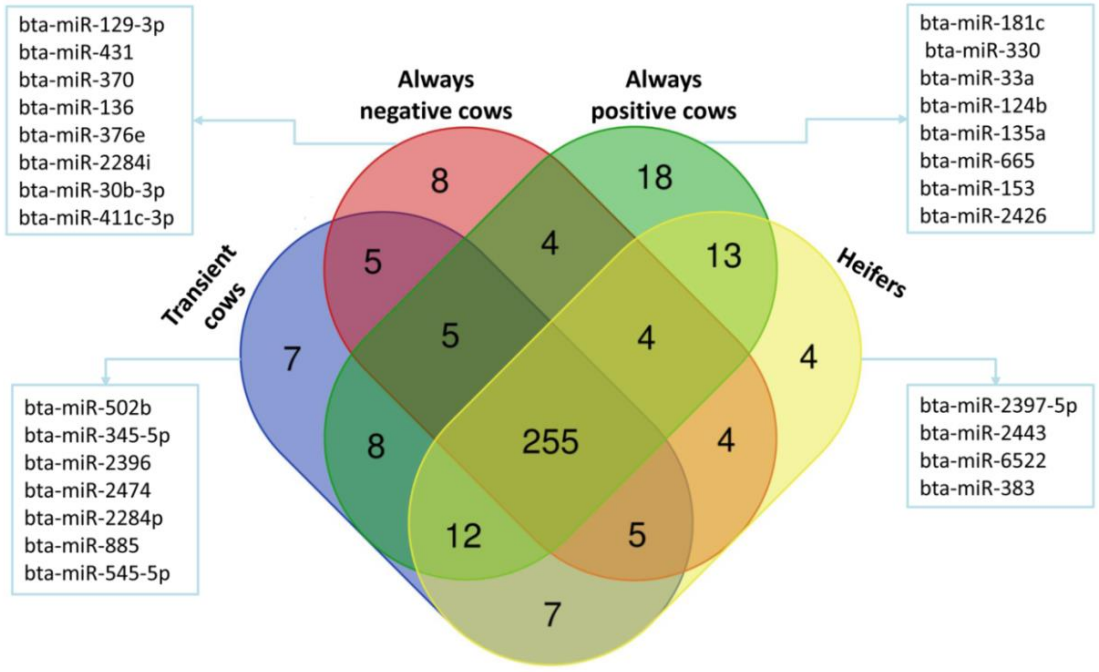

Figure 2.4: Venn diagram showing unique and shared detection of miRNAs in TCs, ANCs, APCs, and heifers. For a miRNA to be considered as detected, we use a read count of at least 1 and above throughout all biological replicates.

2.3.5 Hierarchical clustering of EV-coupled miRNA in cows of different metabolic status

Differential expression analysis of miRNAs recovered from follicular fluid of cows in different metabolic status revealed that cows with different metabolic statuses exhibited different miRNA expression profiles. Pairwise comparisons between APCs, ANCs, and TCs and control heifers determined that the differentially expressed (DE) miRNA profiles of TCs and APCs were relatively similar whereas ANCs exhibited a more distinct DE miRNA profile (Fig. 2.5). Namely, most of the DE miRNAs were downregulated in ANCs while a large proportion of the TCs and APCs DE miRNAs exhibited induced expression compared to control heifers. Comparative analysis of miRNAs in follicular fluid derived from metabolically stressed and non-stressed cows.

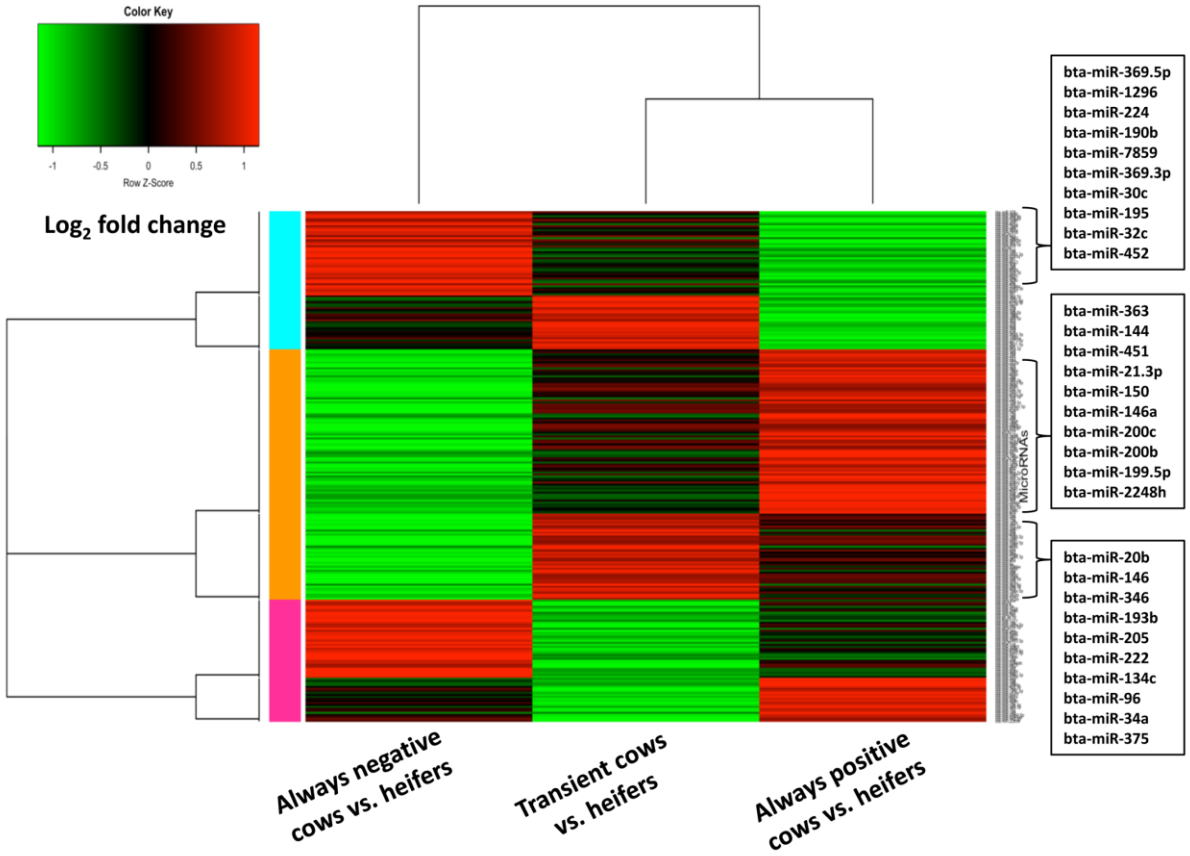

Figure 2.5: Heat map of differentially expressed EV miRNAs in follicular fluid of cows of different metabolic status at weeks 5 and 6 compared to heifers. The log_2 fold change of each miRNA in different pairwise comparisons was used to create the heat map using R-studio package.

In order to identify miRNAs differentially abundant in EVs derived from cows which consistently exhibited negative or positive energy status, the miRNA expression profiles of ANCs and APCs were compared in a pairwise fashion. Interestingly, all six differentially expressed miRNAs were downregulated in cows that persistently remained in metabolic stress conditions compared to cows always in positive energy status (Fig. 2.6). Target prediction of those downregulated miRNAs revealed their involvement in various pathways associated with ovarian function including apoptosis, hippo signaling, TGF-beta signaling, lysine degradation, cell cycle, FoxO signaling, mTOR signaling and others (Fig. 2.6).

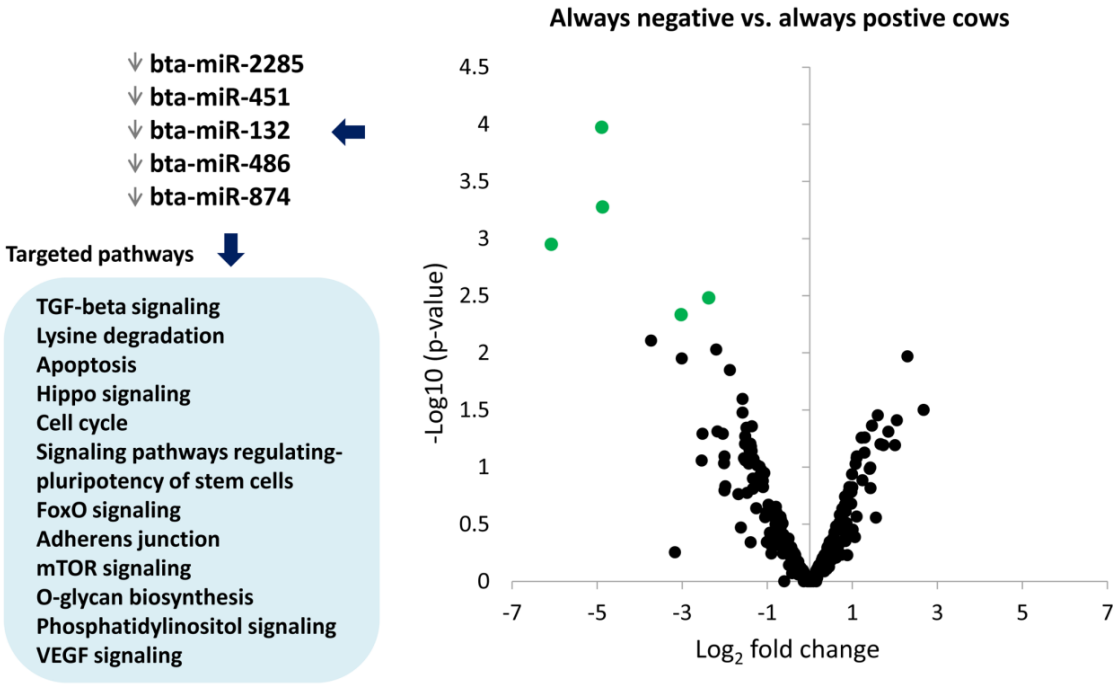

Figure 2.6: Volcano plot of differentially expressed EV-coupled miRNAs in follicular fluid of ANCs compared to APCs and the enriched pathways targeted by downregulated miRNAs. Downward arrows indicate downregulation in expression in ANCs compared to APCs (Details of raw read count and FDRs are presented in Supplementary Table S1).

Furthermore, pairwise comparison of the miRNA expression profiles of ANCs to metabolically unstressed heifers showed differential expression of 37 miRNAs, of which 25 miRNAs were downregulated, and only 12 miRNAs were upregulated (Table 1). Moreover, target prediction and subsequent pathway analysis for downregulated miRNAs revealed pathways involved in different metabolic processes and oocyte follicular growth, including insulin signaling, estrogen signaling, MAPK signaling, vitamin B6 metabolism, fatty acid biosynthesis, fatty acid metabolism, fatty acid elongation, progesterone-mediated oocyte maturation, and others as indicated in the supplementary Fig. 2.S4. Moreover, miRNA-gene network analysis revealed the involvement of candidate miRNAs and their target genes in the interaction network as indicated in supplementary Fig. 2.S6.

Table 2.1: List of differentially expressed EV-coupled miRNAs in follicular fluid of 3ANCs versus heifers

MiRNA name	Normalized TMM read counts		Log_2 Fold change	p value	MiRNA name	Normalized TMM read counts		Log_2 Fold change	p value
	Always negative	Heifers				Always negative	Heifers		
bta-miR-20b	0	21.23	-5.57	0.0037	bta-miR-708	30.51	93.28	-1.62	0.0226
bta-miR-363	0	18.68	-5.34	0.0059	bta-miR-200c	924.02	2805.82	-1.61	0.0011
bta-miR-132	93.73	2719.73	-4.85	0.0001	bta-miR-200b	1370.16	4067.04	-1.57	0.0016
bta-miR-184	174.65	2454.35	-3.84	0.0005	bta-miR-432	279.09	738.26	-1.43	0.0202
bta-miR-451	20.05	228.61	-3.77	0.0001	bta-miR-182	240.47	598.4	-1.31	0.0095
bta-miR-223	125.45	875.5	-2.84	0.0000	bta-miR-191	7774.03	17291.16	-1.15	0.0181
bta-miR-18a	14.1	76.19	-2.66	0.0035	bta-miR-125a	11060.62	5530.14	1.00	0.0252
bta-miR-122	1924.24	12098.39	-2.66	0.0036	bta-miR-26a	14872.37	7174.03	1.05	0.0204
bta-miR-147	10.09	56.61	-2.65	0.0069	bta-miR-423-5p	62976.07	30282.96	1.06	0.0194
bta-miR-21-3p	6.08	37.25	-2.59	0.0291	bta-miR-450b	1624.61	776.18	1.07	0.0142
bta-miR-150	181.84	914.97	-2.37	0.0002	bta-miR-328	455.07	215.61	1.08	0.0209
bta-miR-146a	86.91	376.36	-2.18	0.0014	bta-miR-92a	60915.34	27486.24	1.15	0.0145
bta-miR-138	6.2	38.39	-2.17	0.0121	bta-miR-92b	6096.23	2558.5	1.25	0.0254
bta-miR-193b	4.13	26.48	-2.15	0.0242	bta-miR-6520	117.92	46.65	1.30	0.0288
bta-miR-155	325.07	1375.11	-2.09	0.0006	bta-miR-542-5p	388.66	151.21	1.35	0.0060
bta-miR-142-5p	583.69	2285.45	-1.98	0.0016	bta-miR-1306	393.91	139.79	1.51	0.0056
bta-miR-205	1000.1	3541.22	-1.83	0.0079	bta-miR-424-5p	2015.21	654.03	1.62	0.0006
bta-miR-183	176.76	619.32	-1.82	0.0049	bta-miR-6518	71.36	21.97	1.70	0.0140
bta-miR-206	30.76	109.02	-1.70	0.0151					

Extended list of miRNAs with the corresponding raw read count and FDRs are presented in Supplementary Table S2

2.3.6 EV-coupled miRNAs as predictors of transition from negative energy to positive energy status during early lactation

The follicular fluid EV-coupled miRNA profiles of ANCs and TCs at weeks 5 & 6 was compared to determine candidate miRNAs that may be indicators for the transition to positive energy balance in the later stages of lactation (weeks 9 and 10). In this comparison, miRNAs (i.e., bta-miR-34b, bta-miR-34c, bta-miR-449a, and bta-miR-132) were downregulated in ANCs compared to TCs, which have recovered from their metabolic stress at weeks 9 & 10. Comparison of DE miRNAs between EVs recovered from follicular fluid collected from

transient cows at weeks 5-6 and weeks 9-10 revealed that bta-miR-34b, bta-miR-34c, and bta-miR-449a were upregulated, whereas two miRNAs, bta-miR-451 and bta-miR-592, were downregulated at weeks 5-6.

2.3.7 Identification of EV-coupled miRNAs induced or suppressed by lactation physiology

In order to determine the effect of lactation on EV-coupled miRNA release in follicular fluid, the miRNA expression profiles of APCs versus heifers were compared. Both groups are metabolically unstressed and any differences in miRNA expression would likely relate their different lactation physiologies. Our results revealed that out of 38 differentially expressed miRNAs, 31 miRNAs were upregulated, and only seven miRNAs were downregulated in APCs or lactating cows compared to heifers (Table 2). Target prediction of lactation-induced miRNAs revealed their involvement in various biological pathways, including gap junction, PI3K-Akt signaling, steroid biosynthesis, fatty acid degradation, ErbB signaling, AMPK signaling, sphingolipid signaling, central carbon metabolism, insulin signaling, phosphatidylinositol signaling system, prolactin signaling, MAPK signaling, ras signaling, estrogen signaling, fatty acid metabolism, fatty acid elongation, mTOR signaling, p53 signaling, focal adhesion, Wnt signaling and others as indicated in the supplementary Fig. 2.S5.

Table 2.2: List of differentially expressed EV-coupled miRNAs in follicular fluid of APCs compared to heifers

	Normalized TMM read counts		Log_2 Fold change	p value		Normalized TMM read counts		Log_2 Fold change	p value
MiRNA name	Always positive	Heifers			MiRNA name	Always positive	Heifers		
bta-miR-135a	17.13	0.86	3.72	0.0039	bta-miR-6520	145.68	40.8	1.81	0.0029
bta-miR-2285z	34.14	2.38	3.45	0.0001	bta-miR-6518	66.63	19.22	1.78	0.0111
bta-miR-502a	145.84	16.32	3.09	0.0000	bta-miR-2366	95.69	28.92	1.68	0.0084

bta-miR-449a	170.79	21.4	2.95	0.0240	bta-miR-423-5p	82036.05	26419.48	1.63	0.0011
bta-miR-874	230.75	30.7	2.94	0.0000	bta-miR-335	1834.38	614.55	1.58	0.0292
bta-miR-1291	17.13	2.31	2.92	0.0124	bta-miR-652	310.1	105.7	1.55	0.0035
bta-miR-1246	33974.57	4846.76	2.81	0.0000	bta-miR-671	376.02	129.93	1.53	0.0029
bta-miR-320b	22.21	3.76	2.75	0.0085	bta-miR-2284x	13219.16	4627.82	1.51	0.0028
bta-miR-130a	23627.76	4333.53	2.45	0.0007	bta-miR-26a	17422.87	6256.15	1.48	0.0024
bta-miR-331-5p	32.61	5.64	2.44	0.0031	bta-miR-6517	189.85	70.22	1.46	0.0113
bta-miR-23b-3p	38460.93	7357.16	2.39	0.0000	bta-miR-92b	5913.8	2234.63	1.40	0.0251
bta-miR-107	45710.3	9316.24	2.29	0.0001	bta-miR-296-3p	4994.23	2302.28	1.12	0.0261
bta-miR-362-3p	32.36	5.61	2.28	0.0045	bta-miR-204	44.67	126.49	-1.50	0.0163
bta-miR-873	9540.4	2009.85	2.25	0.0077	bta-miR-194	575.71	1742.56	-1.60	0.0120
bta-miR-320a	148650.4	31784.41	2.23	0.0001	bta-miR-205	895.08	3078.37	-1.78	0.0022
bta-miR-103	44908.49	10770.95	2.06	0.0003	bta-miR-196b	22.33	130.08	-2.53	0.0032
bta-miR-23a	47898.5	11874.35	2.01	0.0001	bta-miR-196a	17.38	152.79	-3.12	0.0003
bta-miR-22-3p	9333.16	2434.79	1.94	0.0003	bta-miR-184	140.03	2137.16	-3.93	0.0000
bta-miR-1839	13301.78	3474.36	1.94	0.0009	bta-miR-365-5p	0	16.04	-5.34	0.0057

Extended list of miRNAs with the corresponding raw read count and FDRs are presented in Supplementary Table S3

After performing pairwise comparisons between ANCs and APCs and control heifers, we then compared the DE miRNA profiles of ANCs and APCs to identify commonly differentially expressed miRNAs. These common DE miRNAs may represent circulatory miRNAs that may have been induced or suppressed due to lactation-induced metabolic stress. Out of the seven common miRNAs, miR-205 and miR-184 were suppressed and miR-92b, miR-6518, miR-6520, miR-26a, and miR-423-5p were upregulated.

2.4 Discussion

In this *in* vivo study, the association between postpartum metabolic physiology and EV-coupled miRNAs expression in the follicular fluid of the antrum follicle has been investigated in Holstein Fresian cows and heifers. Previous studies have shown that increased NEFA and β-hydroxybutyrate concentration in the blood stream of metabolically stressed cows resulted in disturbed follicular microenvironments [34]. This phenomenon of negative energy balance has been evidenced to have a huge negative impact on follicular cell development, quality of

oocytes, and embryo survival. However, the molecular mechanisms associated with metabolic stress in dairy cows are poorly understood. In the present study, we have demonstrated the association between metabolic status and EV-coupled miRNA profiles in the follicular fluid of postpartum cows. These changes could partly signify EV-mediated cell-to cell communication between the oocyte and the surrounding cell in antrum follicles with respect to the molecular messages carried by those vesicles. Moreover, several potential candidate miRNAs have been identified, which are not only indicators of negative energy status, but are potential biomarkers for the transition to positive energy status in cows, as shown in TCs. In order to observe the association between postpartum negative energy balance and EV-coupled miRNA signatures in follicular fluid, we analyzed individual cows' metabolic statuses to get a clear demarcation between the experimental groups with respect to the energy status. Overall, we have detected the presence of high blood serum NEFA levels (0.73 ± 0.0.31mmol /L and high β-hydroxybutyrate (0.78 ± 0.33mmol/L), along with body weight loss and lower overall energy (MJ) during the early stage of lactation for the majority of the cows. According to previous studies, lactating cows with an average concentration of NEFA > 0.55mmol/L [32] and β-hydroxybutyrate > 0.65mmol/L [33] in the blood serum are indicators of negative energy balance. While only one fourth of the cows showed a positive energy balance in early period of lactation, increased blood serum NEFA and β-hydroxybutyrate was evident as sign of clinical metabolic status due to lactation during early postpartum for most of the cows. The majority of cows (66%) showed a negative energy balance during their 5-6 weeks postpartum, but they recovered from their metabolic stress in the later stages of the analysis. However, some of the cows (16 %) continued to exhibit negative energy balances until the later stages of the analysis. Interestingly, a similar proportion of cows showed no sign of metabolic stress throughout the analysis period. These results revealed individual differences between cows in terms of dealing with metabolic stress

during early lactation. Molecular genetic analysis of such divergent groups of cows will allow us to understand the molecular mechanism associated with metabolic stress due to lactation and paves the way for identification of metabolic markers and the development of future therapeutic strategies to tackle negative effects from metabolic stress in dairy cow reproductive efficiency.

The EV isolation methodology used in the present study was evaluated based on the size distribution and morphology of the isolated EVs. Moreover, nanoparticle-tracking analysis revealed the majority of the EVs were as big as 124.8 -147.1nm in diameter, indicating absence of contamination of large vesicles during EV isolation. Following morphological characterization, CD63 and Alix protein markers, which are known to be enriched in exosomes from virtually any cell type, were used to determine the specificity of our EV isolation procedure[35]. We have analyzed EV-coupled small RNAs including miRNA with a read length of 75nt. The read length distribution for miRNAs was observed to peak around 18-23nt. Our result revealed that a huge number of known miRNAs (365) and putative miRNAs (158) were detected in EVs recovered from follicular fluid from follicles > 8mm. The detected miRNAs accounted for 48% of the 762 known bovine miRNAs available in mirbase_20-. Considering that about 70% of the total detected miRNAs were found in all experimental groups it suggests they have housekeeping roles in cell-to-cell communication as mediated by EVs in the follicular environment. The housekeeping role of several miRNAs in ovarian granulosa cells from dominant and subordinate follicle has been reported previously [36]. Moreover, sequence analysis revealed the presence of unique EVs coupled miRNAs in the follicular fluid of cows with divergent metabolic status. As shown in Fig. 4, cows that had negative energy status throughout the experimental period showed exclusive expression of eight miRNAs, five out of which (bta-miR-431, bta-miR-370, bta-miR-136, bta-miR-376e and bta-miR-411c-3p) were found on chromosome 21. A comparable number

of miRNAs have been uniquely detected in transiently negative energy cows and heifers. However, the numbers of uniquely expressed miRNAs were highest in cows that consistently exhibited positive energy balances throughout the experimental period. Four of these miRNAs (i.e., miR-2436-5p, bta-miR-33a, bta-miR-135a and bta-miR-2426) were found on chromosome 5, which suggests their importance in reproduction. A previous study showed that deletion between 25 and 70 Mb on chromosome 5 was associated with decreased reproductive efficiency in female cattle [37]. Two of the uniquely detected miRNAs, bta-mir-135a and bta-mir-2426, are found in the same 25 and 70 Mb region. Moreover, the well-studied miR-181 gene family, which was uniquely detected in APCs, is a cellular metabolic rheostat essential for NKT cell development and regulates homeostasis in NKT cell ontogenesis and lymphocyte development in mice [38]. Also, miR-181a regulates lipid metabolism, as miR-181a transgenic mice have lower body weights and less lipid accumulation compared to wild-type [39]. Therefore, the detection of the miR-181 family exclusively in the cows with consistently positive energy balance may be associated with metabolic homeostasis.

Hierarchal clustering of differentially expressed EV-coupled miRNAs between cows with divergent metabolic statuses revealed a clear association between metabolic stress and the release of EV-coupled miRNAs. As shown in Fig. 4, TCs and APCs have relatively similar expression patterns, while ANCs exhibited reduced expression of a significant number of EV-coupled miRNAs. Previous studies have shown the association between glucose level and expression of miRNAs in human mesenchymal stem cells [40], in which higher glucose levels suppressed the expression of candidate miRNAs. The reduced release of EV-coupled miRNAs in the follicular fluid of metabolically stressed cows in the present study could be associated with reduced glucose concentration in those animals. This has been also evidenced in the direct comparison of always negative versus always positive cows for which a large set

of known and putative miRNAs were found to be globally suppressed in the always negative energy cows. This suggests metabolites in the follicular fluid do have a direct regulatory role in the expression of miRNAs in follicular cells and their subsequent release into the extracellular environment. Even though the mechanism behind the response of the genome to metabolic changes is not fully understood, specialized transcription factors have been documented to be activated in response to metabolic changes and result in gene expression alterations [41]. Hormonal regulation of miRNAs is a well-documented fact in various mammalian species. The expression of miRNAs including miRNA-183, miRNA-132 and miR-122, which are among candidates in the present study, have been reported to be altered after treatment 17ß-estradiol in Rat granulosa cells [42]. Interestingly, early postpartum dairy cows with metabolic stress have been documented to have very law oestradiol concentrations [43]. Therefore, the major suppression of EVs-coupled miRNA in the current study could be associated with the law level of oestradiol and suggests metabolites in the follicular fluid do have a direct regulatory role in the expression of miRNAs in follicular cells and their subsequent release into the extracellular environment. Similarly, supplementation of elevated NEFA concentrations during oocyte maturation in vitro found to alter the expression level of DNMT3A, IGF2R and SLC2A1 genes in day 7 blastocysts [44].

In the current study, the functional relevance of differentially expressed miRNAs was determined based on target prediction and subsequent pathways analysis in which individual or cluster of miRNAs are involved in. Comparative analysis of ANCs vs APCs revealed down regulation of miR-2285, miR-451, miR-132, miR-486 and miR-874 in EVs isolated from follicular fluid of ANCs. In-silico analysis revealed the involvement of those miRNAs in various pathways including TGF-beta signaling pathway (Fig. 5), which are known to be involved in oocyte and embryonic development. Knockdown of TGF-β pathway causes embryonic lethality in mouse[45]. Previously, upregulation of bta-miR-451 was documented in

large and healthy follicles compared to small follicles in bovine [46]. Moreover, in C. elegans, TGF-β and Insulin signaling is believed to regulate the reproductive aging by modulating multiple aspects of the reproductive process, including embryo integrity, oocyte fertilizability, chromosome segregation fidelity, DNA damage resistance, and oocyte and germline morphology [47]. Among the downregulated miRNA, the bta-miR-2284 and bta-miR-2285 families, which encode more than 100 mature miRNAs in the bovine genome, are reported to be expressed in a bovine immune-relevant tissues including CD14+ monocytes, mammary epithelial cells, and others [48]. Moreover, bta-miR-132 is found to be moderately correlated with lactose in lactating cows [49]. Upregulation of miR-132 in mouse granulosa cells reported to promote estradiol synthesis by targeting the cAMP signaling pathway through translational repression of Nurr1 gene [50]. In human follicular fluid, the exosomal miRNAs; miR-132, miR-212, and miR-214 coordinately targeted Phosphatase and Tensin Homologue expression (PTEN) and have been documented to involve triggering meiosis resumption [31]. In addition, the expression of miR-485-5p was reported to be controlled by high glucose levels in humans [40]. The same study showed that overexpression of miR-486-5p induced a premature senescence-like phenotype by inhibiting proliferation of hAT-MSCs and adipogenic and osteogenic differentiation, whereas the reverse was observed by inhibition of miR-486-5p [40].

Massive downregulation of EV-coupled miRNAs were found during comparison of ANCs versus heifers (Table 1). In that comparison, 25 out of 37 differentially expressed miRNAs were suppressed in ANCs, supporting the notion that metabolic stress impairs the release of EV-coupled miRNAs into extracellular space. One of those miRNAs was miR-21, which is a well-studied miRNA known to be involved in folliculogenesis. Upregulation of miRNA-21 in mice was found to be associated with increased cell survival and promotion of ovulation[51]. A linear increase of miR-21 was also found to be involved on controlling maternal-to-

embryonic transition and early development [52]. Therefore, the downregulation of EV-coupled miR-21 in the present study due to metabolic stress may implicate the corresponding reduced expression in the follicular cells, which may impair follicular development. Similarly, elevated expression of miR-20b in bovine cumulus cells increased oocyte maturation and progesterone synthesis by targeting INHBA, MAPK1, PTGS2, PTX3, and EGFR [53].

Comparative analysis of EV-coupled miRNAs in ANCs versus TCs at 5-6 weeks postpartum could reveal potential miRNAs as predictors of cows that will maintain negative energy balance or those that will recover in the later stages of lactation at weeks 9-10 postpartum. In that comparison, all four differentially expressed miRNAs, miR-132, bta-miR-34b and bta-miR-34c from the mir-34 family, and bta-miR-449a were found to be suppressed in cows that remained under metabolic stress throughout the experimental period. Interestingly, miR-132 and miR-34 were highly abundant in the follicular fluid of cows that under-went ovarian hyperstimulation compared to unstimulated ones [54]. This may indicate that metabolically stressed postpartum cows exhibited delayed resumption of ovarian cyclicity that may have been associated with disturbed serum metabolite profiles [55]. Therefore, the candidate miRNAs can be potential indicators of persistent or transient occurrence of metabolic stress in postpartum cows. Future research need to be done to validate this notion in an independent population and further functional studies need to be done to establish these candidate miRNAs as predictors of persistent or transient metabolic stress in dairy cows.

Irrespective of the metabolic status, comparative analysis of APCs and heifers enabled us to identify circulatory miRNAs associated with lactation physiology. Cows under positive energy balance postpartum showed induction of 31 miRNAs compared to unstressed control heifers as indicated in Table 2. Seven miRNAs were differentially expressed in common between APC and ANCs when both compared to heifers. Out of these bta-miR-184 and bta-

miR-205 were downregulated and the other five miRNAs (i.e., bta-miR-26a, bta-miR-423-5p, bta-miR-6518, bta-miR-6520, and bta-miR-92b) were upregulated in both ANCs and APCs compared to heifers. This could be the result of the interaction of metabolism and lactation induced change on the abundance of EV- coupled miRNAs. This could be associated with the nutritional change after parturition in the cows and may have a short-term effect on the follicular microenvironment. Previously high lactation performance has been reported to be associated with high abundance of miRNA-29 expression in dairy cow mammary epithelial cells [56]. Based on this fact and the results of the present study, the lactation physiology of the animals may have a profound effect on the transcriptome of follicular cells and subsequent release of EV-coupled miRNAs into extracellular space. However, further studies need to be done to elucidate the effect of lactation in follicular cells transcriptome and its association with oocyte physiologys.

2.4.1 Conclusion

In conclusion, our results revealed that negative energy balance in postpartum cows is mainly associated with downregulation of EV-coupled miRNAs in follicular fluid, whereas upregulation of EV-coupled miRNAs was associated with positive energy balance in dairy cows. Moreover, lactation induced changes in follicular fluid EV-coupled miRNA profiles in dairy cows regardless of energy status. The divergent expression of EV-coupled miRNAs in follicular fluid of metabolically stressed cows could partly explain the reduced fertility in high yielding dairy cows. Resolving the array of EV-coupled miRNAs in follicular fluid of metabolically divergent cows will undoubtedly extend our understanding on the molecular mechanisms associated with the effect of metabolic stress in ovarian functionality. Further validation of the candidate markers from this study in different population and other stress relevant experimental setup could lead to the development of markers which can be used as foundation to enhance reproductive function or treat ovarian dysregulation.

2.5 Material and methods

2.5.1 Assessment of body weight and overall energy balance

Experimental animal handling was done in accordance with the 2015 German law of animal protection (TierSchG & TierSchVersV). Experimental protocols were approved by the state office for Nature, Environment and Consumer protection of North Rhine-Westphalia, Germany (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Deutschland) with respect to blood sample collections by licensing number 84-02.04.2015.A139 and with respect to Ovum Pick Up (OPU) procedure by licensing number 84-02.04.2014.A500. All animals were kept in the dairy farm of the Frankenforst teaching and research station at the University of Bonn. Holstein Friesian cows (n=30) and heifers (n=8) were used and kept under conditions that enabled feed-intake, milk yield, and body weight data collection from individual cows. The body weight, milk yield, and dry matter intake of individual cows were measured daily for 15 weeks postpartum. Moreover, individual cow dry matter and concentrate intake was measured daily. Following that, the overall energy balance of individual cows was calculated on weekly basis using the following equation: Energy balance (EB) (MJ NEL/d) = Energy intake (EI) - Energy consumption (EC). Whereby: Energy intake (EI): Dry matter concentration of PMR (partial mixed ration) x Intake PMR (kg fresh matter) x energy concentration of PMR (MJ NEL/kg DM) plus concentrate intake (kg) x energy concentration of concentrates (MJ NEL/kg) and Energy consumption (EC): minus energy for maintenance: (0.293 x body weight (BW) 0,75) minus milk energy: (0.39 x fat% + 0.24 x protein% + 0.17 x lactose% + 0.07) x kg milk).

2.5.2 Blood serum metabolite analysis

Blood samples were collected from individual postpartum cows (n=30) at weeks 5, 6, 7, 8, 9 and 10 post-calving and in parallel to ovum pick-up time. Similarly, blood samples were collected from heifers (n=8) during ovum pick-up sessions for five consecutive weeks.

Approximately, 20 ml of blood were collected from all postpartum cows and heifers at each collection time pointinto additive carrier serum separation tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). Blood samples were cooled for 30 min at 4 °C and centrifuged at 2500 xg for 10 min to separate blood serum from the blood cells and clotting factors. Serum samples from individual postpartum cow and heifers were subjected to metabolite analysis, including analysis of nonesterified fatty acids (NEFA) and beta-hydroxybutyrate (β-OHB), in order to determine energy status of experimental animals as previously published in(Frieten *et al.* 2017). Briefly, NEFA concentration was quantified using series NEFA-HR (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's protocol. Seven micro liter of the sample calibrator was added into 300 µl of blood serum and incubated at 37 °C for 7.5 minutes. The concentration of NEFA was measured with a main wavelength of 546 nm and sub-wavelength of 660 nm. Similarly, the concentration of β-OHB was quantified using Ranbut D-3-hydroxybutrate with the BHBA kit (# RB1008) (Randox laboratory limited, United Kingdom) according to the manufacturer's protocol.

2.5.3 In vivo follicular fluid collection using Ovum pick-up

Collection of follicular fluid (FF) from postpartum cows (n=30) and heifers (n=8) was performed using an ovum pick-up (OPU) technique from large follicles with diameters >8mm. the fluid collection was done from postpartum cows at weeks 5, 6, 7, 8, 9 and 10 postpartum. Heifer FF collection was performed for five consecutive weeks. Oocytes were picked under the microscope prior to centrifugation. Follicular fluid was centrifuged at 500 g for 5 min at room temperature to remove cell debris and pellet granulosa cells. Follicular fluid samples were then transported in liquid nitrogen and stored at -80 °c until further analysis.

2.5.4 Isolation of extracellular vesicles from follicular fluid using ultracentrifugation

Prior to pooling FF samples, the metabolic statuses of individual postpartum cows were assessed using three criteria: blood serum metabolite analysis (5-10 weeks), body weight curves (1-15 weeks), and overall energy balance based on dry matter intake (1-15 weeks). Based on these three criteria's, animals were grouped into three phenotypes namely: Transient cows (TCs) (n=20), Always Negative cows (ANCs) (n=5) and Always positive cows (APCs) (n=5) (Fig. 2.2). Follicular fluid samples from cystic ovaries and blood contamination are discarded from the analysis. Follicular fluid samples from TCs at week 5 and 6 (n=11) and at weeks 9 and 10 (n=7) were separately pooled to create three biological replicates. Follicular fluid collected from ANCs (n=2) and APCs (n=2) at weeks 5 and 6 were pooled to generate two biological replicates per group of cows. Follicular fluid from heifers (n=8) was pooled to create three biological replicates from the 5 consecutive weeks. Extracellular vesicles were isolated from a pool of 2ml FF using differential centrifugation followed by ultracentrifugation fitted with an SW 55 Ti Rotor (Beckman Coulter, Germany). Briefly, FF was diluted with an equal amount of PBS-CMF (2 ml). Follicular fluid was centrifuged at 500 xg for 10 minutes to remove cell debris followed by another centrifugation at 6000 xg for 20 minutes to remove apoptotic bodies. The supernatant was transferred to a new tube and centrifuged at 18,000 xg for 30 minutes to remove microvesicles. The supernatant containing extracellular vesicles was transferred to a new 5ml polypropylene centrifuge tube (Beckman Coulter, Krefeld, Germany) and centrifuged at 120,000 xg for 70 minutes to pellet EVs. The EV pellets were re-suspended with 5 ml of PBS and centrifuged at 120,000 xg for 90 minutes. Finally, EV pellets were resuspended in final volumes of 1ml in PBS and stored in -80 ^{O}C until further analysis.

2.5.5 Nanoparticle tracking and electron microscopy analysis of extracellular vesicles

Nanoparticle tracking analysis was performed to determine the concentration and size distribution of isolated extracellular vesicles using Nano Sight NS300 following the manufacturer's protocol (Malvern Instruments, Malvern, UK). Briefly, 25 µl of EV pellet was mixed with 1 ml of PBS-CMF and assembled for five video recordings. Sequenced data were analyzed using NTA version 3.2 and the mean, mode, standard deviation, and concentration of particles were obtained. Furthermore, EVs were morphologically characterized using an electron microscope (Ziess EM109, Carl Zeiss). Briefly, 20µl drops of purified EVs on parafilm were covered by Formvar-carbon-coated grids. Five minutes later, the Formvar-carbon-coated grids were washed using drops of PBS-CMF and incubated in 30µl of 2 % uranyl acetate. Grids were washed with drops of PBS-CMF and examined under an electron microscope.

2.5.6 Western blot analysis

Detection of two EVs protein markers CD63 [25,58] and Alix [26,58] was performed by western blotting. The extraction of EV protein was performed using radioimmuno precipitation assay buffer (RIPA) according to the manufacturer´s instruction (Sigma-Aldrich, Germany). Briefly, isolated EV pellets were resuspended in 100 µl of RIPA buffer and vortexed for 15 seconds. Briefly, isolated EV pellets were suspended in 100µl of RIPA buffer. About 30µg of extracted protein was loaded on to an SDS gradient gel, electrophoresed, and blotted to nitrocellulose membrane using Biorad Pac 3000 transblot SD semi-DRY transfer cell (BioRad, USA). Furthermore, the membrane was incubated in a 10ml blocking buffer for 1hr at room temperature followed by incubation overnight with the primary antibody at 4 ºC. For this Primary antibody of CD63 and Alix (System biosciences, Palo Alto, Canada) were diluted with blocking solution in a ratio of 1:300. Afterwards, the membrane was incubated for 1 hr with the secondary antibody of Goat Anti-Rabbit HRP (System biosciences, Palo

Alto, Canada) with a dilution of 1:8000 in blocking solution. The membrane was further incubated for 5 minutes using an equal amount of peroxide and luminol/enhancer solution (BioRAD, USA). The bands were visualized using the ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, USA).

2.5.7 Isolation of total RNA including miRNAs

Isolation of EV total RNA enriched for miRNA was done using an exosomal RNA isolation kit (Norgen, Canada) according to the manufacturer´s protocol. Briefly, isolated EVs suspended in 400 µl PBS-CMF were lysed with 600µl of lysis buffer and 75µl of lysis additive and incubated for 10 minutes at room temperature. After incubation, 500µl of absolute ethanol was added to the solution and transferred to a mini spin column followed by centrifugation at 3,300 ×g and washing steps. Total RNA was eluted in 32µl of elution solution. Prior to downstream applications, the concentration and integrity of the total RNA were assessed using NanoDrop 8000 spectrophotometer (NanoDrop Technologies, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, CA), respectively.

2.5.8 Next generation sequencing and data analysis

Illumina-based next generation sequencing (NGS) of small RNA and miRNA was performed (Qiagen, Germany). For each sample, a total concentration of 160ng with the maximum input volume of 5µl was processed using the QIAseq miRNA Library Prep kit according to the manufacturer's protocol. Sequencing was performed on an Illumina NextSeq500 sequencing instrument (Qiagen, Germany). The quality of raw FASTQ file was assessed using FastQC version 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Data analysis was performed according to the XploreRNA pipeline (Qiagen, Germany). Briefly, adaptors were trimmed from the raw sequences using Cutadapt (1.11) and mapped against the indexed bovine reference genome (UMD3.1) using Bowtie2 (2.2.2) software with a criterion of one mismatch in the first 32 bases of the reads without insertion or deletion. Sequence reads,

which were aligned to the bovine reference genome, were then used for annotation against bovine precursor miRNAs and matured miRNAs in the miRbase database, release 20. Normalization of raw expression data was done using the trimmed mean of M-values (TMM method of normalization), which is based on log-fold and absolute gene-wise changes in expression levels between samples. Differential expression analysis was performed on the TMM normalized expression values using EdgeR statistical software package (Bioconductor, http://www.bioconductor.org/). A miRNA with a $\log_2$ fold change of $1 \geq \log_2 \leq -1$p-value < 0.05 and false discovery rate of ≤ 0.25 was considered as statistically significant. The potential pathways enriched by differentially expressed miRNAs were analyzed using DIANA-miRPath v3 (http://snf-515788.vm.okeanos.grnet.gr/). Moreover, miRNA-gene network analysis was performed using ingenuity pathway analysis (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/).

2.5.9 Data Availability

The authors confirm that all data underlying the findings are fully available. Sequence data files are available in GEO database with accession number: GSE129367.

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Author Contribution statement

Conceptualization: DT, KS, MH; Design of the work: TH, DT, KS MH.; Data analysis: TH, MP, CD, ET; Interpretation of data: TH, DT, KS, MH; Supervision: DT, KS; Project administration: TH, DT, KS, MH, CN; Funding acquisition: DT, KS; Resource: SG, DSW, FR, MSZ, DT, CN.

Competing interests

Authors declare that there is no conflict of interest.

Chapter 3: Extracellular vesicle-coupled miRNAs in blood serum as a potential noninvasive indicator of metabolic status in lactating cows

(Manuscript under preparation)

Extracellular vesicle-coupled miRNAs in blood serum as a potential noninvasive indicator of metabolic status in lactating cows

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3.1 Abstract

Negative energy balance during early lactation is the leading cause of fertility reduction in dairy cows. Extracellular vesicles are evolutionarily conserved communicasomes and fitted to carry various molecules including microRNAs. The current study aimed to investigate the effect of postpartum metabolic stress on the dynamics of extracellular vesicle-coupled miRNAs content in blood serum as a potential noninvasive indicator of metabolic stress in Holstein-Friesian cows. For this, blood samples were collected from postpartum dairy cows (n=30) from week 5-10 of postpartum and heifers (n=8) for continuous 5 weeks. The energy balance status of postpartum cows was assessed based on their blood metabolites (non-esterified fatty acids and β-hydroxybutyrate), body weight loss, and overall energy output. Based on these criteria, cows were categorized as transient (TCs), always negative (ANCs), and always positive (APCs) cows prior to sample pooling. Blood serum samples were pooled at week 5 & 6 and at week 9-10 and subjected to extracellular vesicle isolation. Extracellular vesicles were isolated using ultracentrifugation. The expression profiling of extracellular vesicle-coupled miRNAs was performed using Illumina-based Next-generation sequencing. Our result revealed the presence of 179 known and 118 novel miRNAs across all experimental groups. Hierarchical clustering of miRNAs showed that downregulation of most miRNAs in metabolically stressed cows compared to non-stressed ones. Pairwise comparison of differentially expressed extracellular vesicle-coupled miRNAs in both ANCs and TCs at week 5&6 vs their control heifers revealed massive downregulation. Out of these, 20 miRNAs (17 down and 3 up) were showing similar patterns as in the ANC and TCs compared to heifers and could be a potential indicator of metabolic stress. Further comparison of ANCs vs APCs revealed downregulated (miR-375, miR-145, and miR-122) and upregulated miR-133a, miR-184, and miR-9-5p). Comparison between ANCs vs TCs revealed upregulation of all differential expressed miRNAs and could be a potential indicator

of recovery. In conclusion, the downregulation of extracellular vesicle-coupled miRNAs in blood serum is associated with major downregulation of EVs coupled miRNAs and could be used as potential indicators of metabolic stress in dairy cows. The target prediction of the downregulated miRNAs showed to target different pathways involved in metabolism, lactation and immunological.

3.2 Introduction

Extensive selection for milk yield in the last few decades has revolutionized the dairy industry. However, the increase in milk production capacity of dairy cows had reported to be associated with reduced fertility due to the negative consequences of high milk production. A long term study showed that the pregnancy rate in cattle has declined from 50–60% in the 1970s to 35–45 % in 2000 (Hansen *et al.* 2004). Currently, low fertility is reported as a leading cause of poor productivity that limits the target of having one calf per cow per year (Ioannidis & Donadeu 2016). One of the key problems is associated with the fact that most dairy cows go through the period of negative energy balance during early lactation and increases time to first ovulation and first breeding (Royal *et al.* 2000; Butler 2003; McGuire *et al.* 2004; van Knegsel *et al.* 2007). The term "negative energy balance (NEB)" in high producing dairy cows is defined as a condition during in which the energy intake and requirements for a lactation and maintenance is higher than the total energy intake and the animals use additional energy from body energy reserves to meet their energy deficient (Bauman & Currie 1980). The mobilization of body fat reserves to compensate the shortage of glucose causes the production of high concentration of deleterious metabolites such as non-esterified fatty acids (NEFA) and b-hydroxybutyrate (β-BHB) in the blood (van Knegsel *et al.* 2007; O'Doherty *et al.* 2014; Fatima *et al.* 2014). Moreover, circulating insulin-like growth factor-I (IGF-1) declined sharply in the first week after calving (Llewellyn *et al.* 2007). The metabolic disturbance coupled with enrichment of the deleterious metabolites in

the blood has been reported to be evident in the follicular fluid of the dominant follicle (Leroy *et al.* 2008b) and uterus (Wathes *et al.* 2007). As a consequence, although the resumption and growth of first dominant follicle and its successive number of subordinate follicles as early as 10 days of postpartum, they generally fail to ovulate and rather undergo atresia (Crowe *et al.* 1998a; Crowe *et al.* 2014b).

In multicellular organisms, long and short distance communications can take in multiple forms. Among these, live cells secrete nano-sized extracellular vesicles that deliver signals in to the extracellular space to reach the target cells (Cocucci & Meldolesi 2015). Extracellular vesicles (EVs) are secreted either constitutively or in a regulated manner as tool for intercellular communication (Choi *et al.* 2013). EVs are evolutionary conserved among unicellular and multicellular organisms, a membrane-bound complex cargo molecules and secreted by most types of cells based on the physiological status or as response to specific stimuli such as stress, pathological situations, necrotic debris (Thery *et al.* 2009, Tannetta *et al.* 2014). EVs are composed of different populations of vesicles such as apoptotic bodies (1 µm - 5 µm), microvesicles (100 nm -1 µm), and exosomes (30 - 100 nm). They have been found in almost all biological fluids including blood serum (Caivano *et al.* 2014), blood plasma (Panteleev *et al.* 2017), nasal mucosal (Qiu *et al.* 2012a), cerebrospinal fluid (Saman *et al.* 2012b), breast milk (Admyre *et al.* 2007a), saliva (Ogawa *et al.* 2008a), umbilical cord blood (Li *et al.* 2008), urine and amniotic fluid (Keller *et al.* 2007), follicular fluid (Sohel *et al.* 2013b), semen (Beach *et al.* 2014b) and possibly others.

MicroRNAs are emerged as promising diagnostic biomarkers with high potential in a clinical application (Ioannidis & Donadeu 2016). Circulating miRNAs are stable in the circulation and resistant to RNAses digestion, extreme pH, high temperatures, extended storage, and multiple freeze-thaw cycles and could be used as good biomarkers (Baldassarre *et al.* 2017). Previously, plasma miRNA levels were evidenced to be associated with aging and longevity,

milk production and composition, milk somatic cell count, fertility and lameness (Ioannidis *et al.* 2018). MicroRNAs have been linked to several metabolic pathways such as cholesterol and fatty acid metabolism, pancreatic islet function, and glucose metabolism. Change on the expression of miRNAs has reported associating with lifespan (Lee *et al.* 2017), autoimmunity and disease progression impairment (Snowhite *et al.* 2017). In livestock, circulating miRNA expression profiles have been documented to be altered in response to change in caloric restriction, parasitic infection, pregnancy, and oestrus (Ioannidis *et al.* 2018). Different studies showed that, NEB has brought changes to the dynamic concentration of blood and follicular fluid metabolites and this change is associated with bad fertility. Therefore, we hypothesized that NEB physiology during early lactation affects the dynamics of EV-coupled miRNAs in blood serum and could be used as a potential indicator of metabolic stress in dairy cows. Therefore, the present study was carried out to investigate the association of EVs-coupled miRNA in blood serum obtained from metabolically divergent lactating cows and heifers.

3.3 Material and methods

3.3.1 Blood collection from postpartum cows and heifers

Experimental animal handling was done in accordance with the 2015 German law of animal protection (TierSchG & TierSchVersV). Blood sample collection protocol was approved by the state office for Nature, Environment and Consumer Protection of North Rhine-Westphalia, Germany (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Deutschland) with licensing number 84-02.04.2015.A139. All experimental animals were kept in teaching and research station dairy farm at Franknfrost, University Of Bonn, Germany. For this experiment, multiparous postpartum Holstein Friesian cows (n=30) and nulliparous Holstein Friesian heifers (n=8) were used. Blood samples were collected from all cows at week 5, 6, 7, 8, 9, and 10^{th} of postpartum period. Similarly, blood samples

from heifers were collected for five consecutive weeks. About 20 ml of blood sample was collected from each cow per collection time point using 18 gauge needles connected with 10 ml of syringe in to additive carrier serum separation tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). After collection, whole blood was cooled for 30 minutes at 4^{o}c and centrifuged at 2500 xg for 10 min to separate the blood serum. Blood serum was transfer to 2 ml of cryostat tube and kept at -80°C for the next step.

3.3.2 Body weight and energy balance assessment of individual cows

The body weight and dry matter intake of individual cows were measured daily for 15 weeks postpartum. The overall energy balance of individual cows was assessed based on their daily dry matter and concentrate intake. Afterwards, the overall energy balance was calculated on daily basis using the following equation: Energy balance (EB) (MJ NEL/d) = Energy intake (EI) - Energy consumption (EC). Whereby: Energy intake (EI): Dry matter concentration of PMR (partial mixed ration) x Intake PMR (kg fresh matter) x energy concentration of PMR (MJ NEL/kg DM) plus concentrate intake (kg) x energy concentration of concentrates (MJ NEL/kg), Energy consumption (EC): minus energy for maintenance: (0.293 x body weight (BW) 0,75) minus milk energy: (0.39 x fat% + 0.24 x protein% + 0.17 x lactose% + 0.07) x kg milk). For both body weight and overall energy balance graphs were generated using mean ± standard deviation of all cows GraphPad prism 5.

3.3.3 Blood serum metabolite analysis

Analysis of two blood serum metabolites; NEFA and β-OHB was performed using commercial kits in order to determine the energy status of experimental animals as previously published (Frieten *et al.* 2017). Briefly, NEFA concentration was quantified using series NEFA-HR (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's protocols. Seven microliters sample calibrators were added to 300 µl of blood serum and incubated at 37 °c and 7.5 minutes. The concentration of NEFA was measured with the main

wavelength of 546 nm and sub-wavelength 660 nm. The concentration of β-OHB was quantified using Ranbut D-3-hydroxybutrate using BHBA (# RB1008) kits (Randox laboratory limited, United Kingdom) according to the manufacturer's protocols.

3.3.4 Recovery of extracellular vesicles from blood serum samples

Based on the above three criteria, postpartum animals were grouped into three categories namely: Transient cows (TCs) (n=20), Always Negative cows (ANCs) (n=5) and Always positive cows (APCs) (n=5). Afterward, blood serum samples from TCs at weeks 5 and 6 (n=11) and at weeks 9 and 10 (n=7) were separately pooled to create three biological replicates. Blood serum collected from ANCs (n=2) and APCs (n=2) at weeks 5 & 6 were pooled to generate two biological replicates per group of cows. Blood serum from Heifers (n=8) was used to generate three biological replicates. Extracellular vesicle isolation was performed using differential centrifugation followed using ultracentrifugation procedure. Briefly, a pool of 12 ml blood serum was used per replicate and diluted with an equal amount of PBS-CMF prior to centrifugation. Diluted blood serum samples were centrifuged at 500 xg for 10 min to remove cell debris followed by another centrifugation at 6000 xg for 20 min to remove the apoptotic bodies. Thereafter, the supernatant was transferred into a new tube and centrifuged at 18,000 xg for 30 min to remove large vesicles as a pellet. Afterward, the supernatant containing was transferred into a new 25 ml polypropylene centrifuge tube and centrifuged at 130,000 xg for 70 min to pellet the EVs. The EVs pellets were resuspended in 25 ml PBS-CMF and washed three times by centrifugation for 70 min at 130,000 xg. After washing steps, EVs pellets were resuspended in final volumes of 2 ml in PBS-CMF and stored at -80 ^{O}C for further characterization and total RNA isolation.

3.3.5 Nanoparticle tracking analysis of blood serum extracellular vesicles

Nanoparticle tracking analysis of extracellular vesicles was performed to determine the concentration and the size distribution of the isolated extracellular vesicles using Nano Sight

NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK). Briefly, 25 µl of EVs pellet was suspended in 975 ml of PBS and used for five recording videos. Recorded videos were analyzed using NTA version 3.2 to generate the mean, mode, standard deviation, and concentration of particles.

3.3.6 Transmission electron microscopy analysis of blood serum extracellular vesicles

EV, morphological characterization was performed using a transmission electron microscope (Ziess EM109, Carl Zeiss). Briefly, 20 µl drops of purified EVs on parafilm were covered by Formvar-carbon-coated grids. Five minutes later, the Formvar-carbon-coated grids were washed using drops of PBS-CMF and incubated in 30 µl of 2 % uranyl acetate. Grids were washed using drops of PBS-CMF and examined under an electron microscope.

3.3.7 Western blot analysis

Molecular characterization of blood serum EVs was done using western blotting to detect the protein markers. Detection of two EVs protein markers CD63 (Sohel *et al.* 2013; Saeed-Zidane *et al.* 2017) and Alix (Navakanitworakul *et al.* 2016; Saeed-Zidane *et al.* 2017) was detected. For that, extraction of EV protein was performed using radioimmuno precipitation assay buffer (RIPA) (Sigma-Aldrich, Germany). About 30 µg of extracted protein was loaded on to an SDS gradient gel, electrophoresed, and blotted to nitrocellulose membrane using Biorad Pac 3000 transblot SD (Bio-Rad, USA). Afterward, the membrane was incubated in 10 ml blocking buffer for 1 hr at room temperature followed by incubation overnight with primary antibody at 4 ºC. For this Primary antibody of Alix and CD63 (System biosciences, Palo Alto, Canada) were diluted with blocking solution at a ratio of 1:300. Afterward, the membrane was incubated with secondary antibody Goat Anti-Rabbit HRP (System biosciences, Palo Alto, Canada) for 1 hr with a dilution of 1:8000 in blocking solution. The membrane was further incubated for 5 minutes using an equal amount of peroxide and

luminol/enhancer solution (Bio-Rad, USA). The bands were visualized using the ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, USA).

3.3.8 Isolation of total RNA enriched with miRNA RNAs

Isolation of EVs total RNA enriched with miRNAs was done using the exosomal RNA isolation kit (Norgen, Canada) according to the manufacturer's protocol. Briefly, isolated suspended EVs pellet lysed with 600 µl of lysis buffer and 75 µl of lysis additive and incubated for 10 min at room temperature. After incubation, 500 µl of absolute ethanol was added to EVs lysate and transferred to the mini spin column for centrifuged at 3,300 xg. After washing steps, the total RNA including miRNA was eluted using elution solution. Prior to downstream applications, the concentration and integrity of the total RNA were assessed using NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Germany) and Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), respectively.

3.3.9 Small RNA library preparation and next-generation sequencing

Illumina based next-generation sequencing platform library preparation was done using QIAseq miRNA Library Prep kit using NextSeq500 according to the manufacturer protocols (Qiagen, Heliden, Germany). Briefly, each samples total RNAs with a concentration of 160 ng along the QIAseq miRNA workflow. Afterward, from each sample of total RNAs with a concentration of 160 ng was used with maximum input 5 µl in volume and went along the QIAseq miRNA workflow and converted into microRNA NGS libraries. Adapters containing UMIs were ligated to the RNA. Then RNA was converted to cDNA and amplified using PCR (22 cycles). Library preparation quality control was performed using Bioanalyzer 2100 (Agilent) and TapeStation 4200 (Agilent). Based on the quality of the inserts and the concentration measurements the libraries were pooled in equimolar ratios. The library pool(s) were quantified using the qPCR ExiSEQ LNA™ Quant kit (Exiqon). The library pool was then sequenced on a NextSeq500 sequencing instrument according to the manufacturer's

instructions. Raw data were demultiplexed and FASTQ files for each sample were generated using the bcl2fastq software (Illumina inc.).

3.3.10 Data analysis

Sequence data analysis was performed using the XploreRNA pipeline (Qiagen, Heliden, Germany). The quality of the FASTQ data was checked using the FastQC Fastqc tool version 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Average base quality, Average base quality, and Spike-in QC were analyzed. Furthermore, the adaptor trimming of the sequence read was performed by removing the library and sequencing adapters (referred to as trimming). Trimming of the raw sequence was done using Cutadapt (1.11) and mapped against the indexed bovine reference genome (UMD3.1). Mapping of the reads sequences against the reference genome was performed using Bowtie2 (2.2.2) software. Mapping to the genome was restricted to one mismatch in the first 32 bases of the reads without insertion or deletion. This sequence reads aligned to the bovine reference genome were then blasted against the bovine precursor and matured miRNAs in miRbase database release-20. Differential expression analysis was performed using the EdgeR statistical software package (Bioconductor, http://bioconductor.org/). Normalization of all samples was done after the trimmed mean of M-values method based on log-fold and absolute gene-wise changes in expression levels between samples (TMM normalization). MicroRNAs with $\log_2$ fold change $\left| 1 \right|$, p-value <0.05 and false discovery rate of ≤ 0.3 were considered as statistically significantly differentially expressed. The analysis of commonly and uniquely expressed miRNA was presented in Venn diagram using the software of bioinformatics (psb.ugent.be/webtools/Venn/).

3.3.11 Target gene prediction

Target genes of the individual differentially expressed of individual miRNAs were predicted using the online prediction tool; Targets can software (http://www.targetscan.org/vert_72/).

Due to the fact that, miRNAs are functionally conserved across mammalian species and the limited number of validated targets for bovine, we use the list of target genes from human. Prediction of enriched pathways was performed using network analyst software (https://www.networkanalyst.ca/).

3.4 Results

3.4.1 Characterization of energy balance status of the dairy cows and heifers

To investigate the metabolic status of experimental animals; the NEFA and beta-hydroxybutyrate were analyzed as serum metabolites weekly. It is well established that, cows in the state of negative energy balance have high concentrations of NEFA (> 0.55 mmol/L) (Fenwick *et al.* 2008)' and ß-BHB (> 0.65 mmol/L) (Girard *et al.* 2015). Based on blood serum metabolite analysis, lactating cows were categorized into three groups. The majority of the lactating cows (20 out of 30) were 'transient cows (TCs)' in their negative energy balance profile with high concentration of NEFA and ß-BHB concentration of 0.82 ± 0.32 mmol/L, and 0.85 ± 0.36 mmol/L, respectively at week 5 and recovered at week 10 with NEFA to 0.41 ± 0.22 mmol/L and BHO to 0.52 ± 0.10 mmol/L (Table 3.1). The second group of cows (5 out of 20) were found to be 'always negative (ANCs)' which had a high concentration of NEFA (0.69 ± 0.08 to 1.05 ± 0.06 mmol/L) and ß-BHB (0.78± 0.17to 0.98 ± 0.02 mmol/L) during the experimental period. The third group of cows (5 out of 20) was found to be 'Always positive cows (APCs)' which revealed lower concentration of NEFA (0.40 ± 0.10 to 0.26 ± 0.19 mmol/L) and ß-BHB (0.53 ± 0.15 to 0.42 ± 0.17 mmol/L) during the experimental period (Table 3.1). In case of heifers, the blood serum concentration of these metabolites was as low as 0.07 ± 0.03 to 0.19±0.13 mmol/L for NEFA and 0.38 ± 0.14 to 0.46 ± 0.05 mmol/L for ß-BHB during the experimental period (Table 3.1).

Table 3.1: Weekly based quantification of blood serum metabolites in cows and heifers (in mmol per liter)

Metabolic status	Mean and SD	Week5		Week 6		week 7		week 8		Week 9		Week 10	
		NEFA	BHO	NEFA	BHO	NEFA	BHO	NEFA	BHO	NEFA	BHO	NEFA	BHO
TCs (n=20)	Mean	0.82	0.85	0.59	0.7	0.65	0.63	0.39	0.73	0.34	0.54	0.41	0.52
	SD	0.32	0.36	0.44	0.29	0.33	0.19	0.19	0.24	0.12	0.08	0.22	0.1
ANCs (n=5)	Mean	0.69	0.78	0.76	0.9	0.87	0.71	0.59	0.88	0.96	0.61	1.05	0.98
	SD	0.08	0.17	0.28	0.28	0.13	0.28	0.24	0.54	0.15	0.06	0.06	0.02
APCs (n=5)	Mean	0.4	0.53	0.52	0.48	0.35	0.54	0.36	0.51	0.5	0.43	0.42	0.42
	SD	0.1	0.15	0.29	0.1	0.11	0.13	0.17	0.1	0.32	0.11	0.19	0.17
Heifers:5 weeks: (n=8)	Mean	0.19	0.38	0.16	0.42	0.28	0.39	0.07	0.5	0.07	0.46		
	SD	0.13	0.14	0.08	0.05	0.13	0.05	0.03	0.06	0.03	0.05		

All measurements are in millimole per liter

In addition to metabolic profiles, cow's body weight measurement was performed for the first 15weeks postpartum period. Body-weight curve assessment of individual cows for consecutive 15 weeks of postpartum time revealed that the majority of the cows were found to lose their body weight as early as week 2 postpartum and recovered during or after weeks 7-8. The remaining cows were either to lose continuously or gain their body weight continuously (Chapter 2 Supplementary Figure-2.S1). Furthermore, measurement of individual cow's energy status was performed based on dry matter intake and energy expenditure for milk yield and maintenance. Our result showed that two-thirds of them were at negative energy balance during the early weeks and continuously recover after week 7-8 (representative figure in Chapter 3 Supplementary Figure-1.S1). The remaining one-third of the cows were either continuously remain at negative or continuously remain at positive energy balance (Chapter 2-Supplementary Figure-2.S2).

Based on the combination of metabolic profiles, body weight curve, and energy balance between weeks 5 and 10 post-calving, all experimental cows were categorized into three groups: 1) Transient cows (TCs): cows, which showed negative energy balances at weeks 5-6 and recovered at weeks 9-10 by showing positive energy balance; 2) Always negative cows

(ANCs): cows that showed negative energy balance at all times between weeks 5 and 10 postpartum, and 3) Always positive cows (APCs): cows that showed positive energy balance through the experimental period as illustrated in Fig. 3.1. Afterward, the blood serum samples were pooled based on the metabolic physiology status at two-time points; week 5- 6 and at week 9-10 of postpartum. All the heifers were at positive energy balance at all time points of the blood collection.

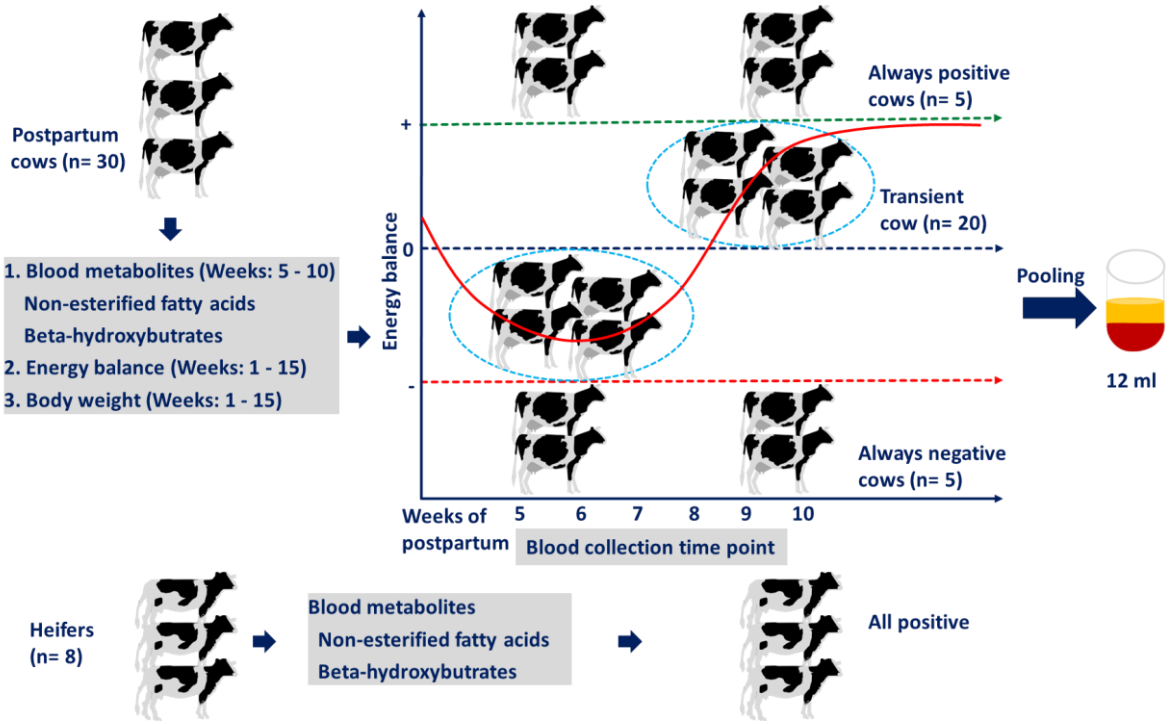


Figure 3.1: Summary of different categories of cows based on energy status as determined from blood serum metabolites, energy balance, and body weight curve of individual animals**.**

3.4.2 Morphological and molecular characterization of EVs recovered from blood serum

To examine the morphology of EVs, a representative EVs morphological characterization was done from all metabolic status cows using a transmission electron microscope as shown in Fig. 3.2A. Furthermore, the concentration and the size distribution of EVs were quantified using a nanoparticle tracking system. The size of the EVs was found to be within the range of

113.4 ± 0.9 to 141.9 ± 0.7 nm in diameter (a representative image is indicated in Fig. 3.2B) with a concentration of between 1.07e ± 09 and 4.39e ±08 particles/ml as sown in Fig. 3.2B. Different previously established EVs markers proteins of the tetraspanin CD63 and Alix were used for protein characterization. Our result revealed that the presence of those protein markers in all sample shows the specificity of EVs isolated from blood serum (Fig. 3.2C). Furthermore, our EVs-coupled small RNA analysis using the electropherogram showed the presence of small RNAs. Further, EVs RNA quality assessment showed the absence of 18s and 28s bands suggesting the absence of cellular RNA contamination (Fig.3.2D).

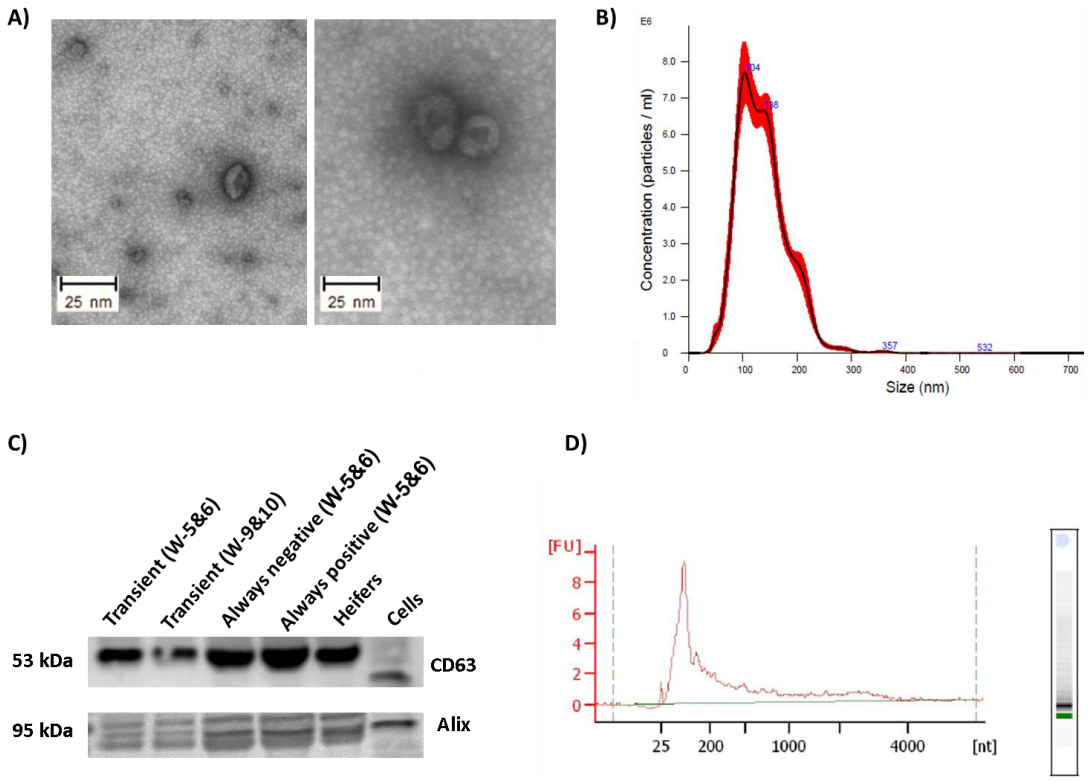


Figure 3.2: Characterization of EVs recovered from blood serum of cows and heifers. A) Electron microscope image of EVs and the red arrow shows the EVs morphology, B) A representative nanoparticle tracking analysis of EVs, C) Detection of EV protein markers and D) A representative electropherogram image of EVs RNA quality analysis using Agilent bioanalyser.

3.4.3 Sequence quality and mapping of RNAseq data from different small RNA samples

The quality of the NGS sequence data samples revealed that, the average read quality the sequencing data to be with a Q-score of above 30 for all samples. Moreover, the average base quality assessment revealed that a Q-score for all samples was above 30 (> 99.9% correct) an indicator of high-quality data for all the samples. An average of 12.2 million reads was obtained for each sample and the average genome-mapping rate was 16.8%.

3.4.4 Detection of EV-coupled miRNAs in blood serum of different metabolic status cows

Illumina based next-generation sequencing results showed that, detection of 179 known miRNAs and 118 novel miRNAs across all the samples. The number of all commonly and uniquely detected miRNAs in TCs, ANCs, APCs, and heifers are indicated in Fig. 3.3.

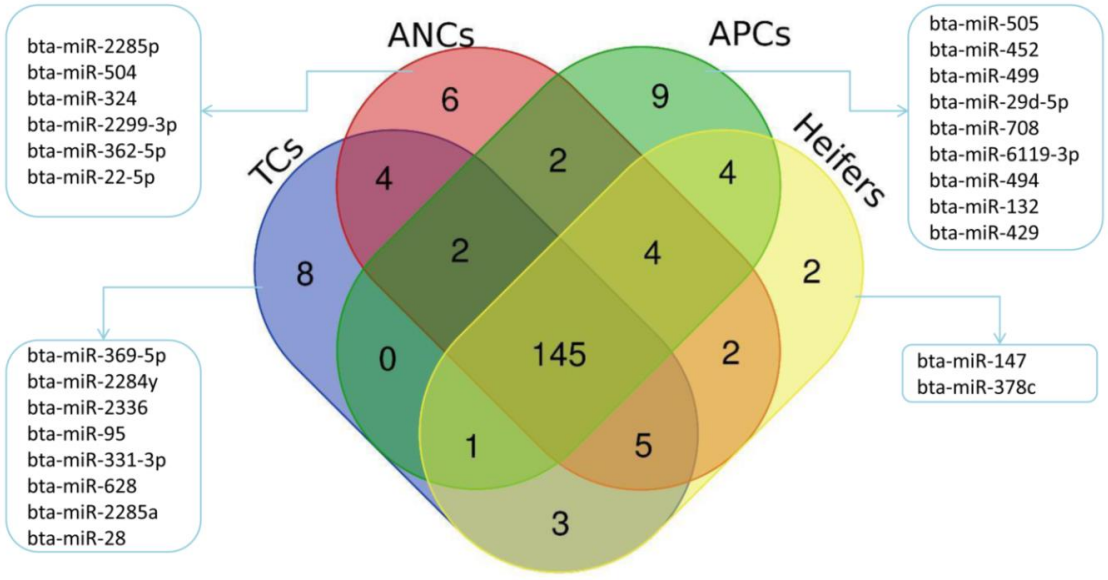


Figure 3.3: A Venn diagram showing the commonly and exclusively detected EV-coupled miRNA among ANCs, TCs, and APCs at week 5 and 6 and heifers. For a miRNA to be

considered as detected, we use a read count of at least 1 and above across all biological replicates.

3.4.5 Hierarchical clustering of EV-coupled miRNAs in the blood serum of cows with different metabolic status

In order to evaluate the correlation between the clustering of individual miRNAs and the corresponding metabolic status, we performed the two-way hierarchical clustering of miRNAs for all individual biological samples of; TCs (week 5-6), TCs (week 9-10), ANCs (week 5-6) APCs (week 5-6) and heifers (Fig. 3.4). The result revealed that most of the miRNAs clustered showed that the APCs and heifers. Unlikely, TCs and ANCs samples were clustered near to each other and the majority of the miRNAs were downregulated.

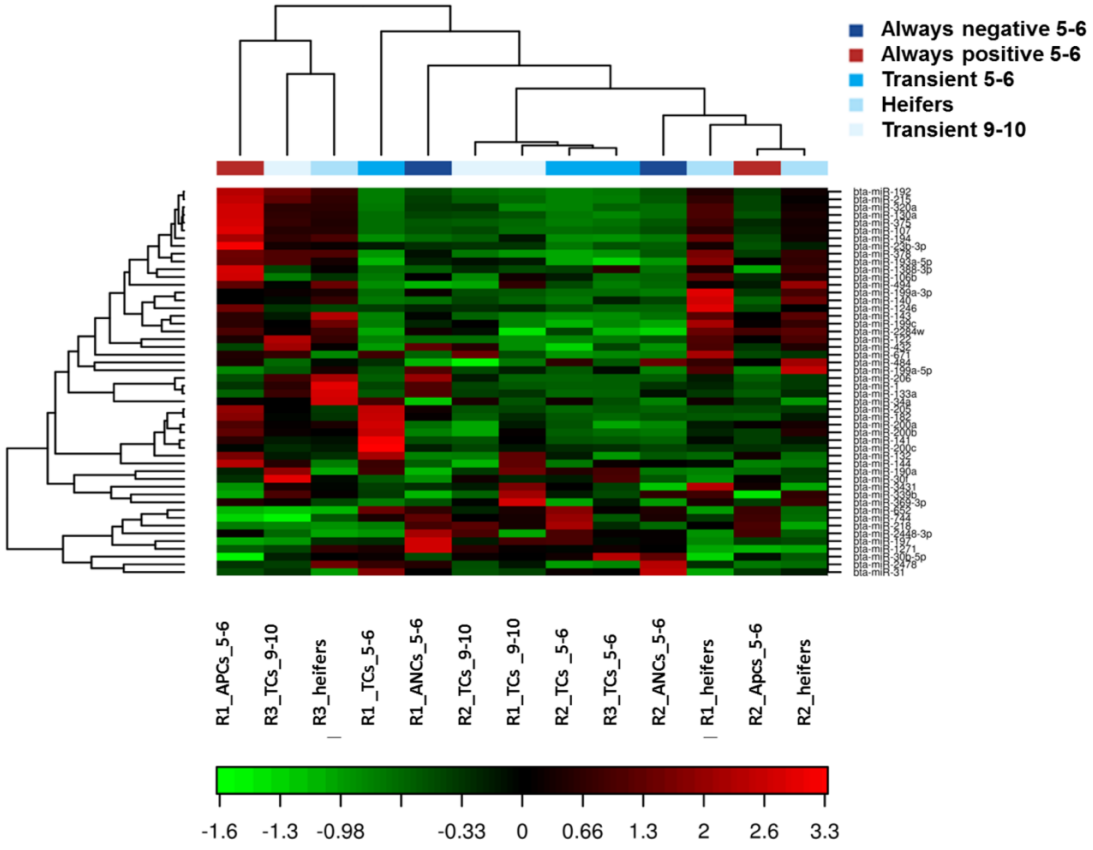


Figure 3.4: Two-way hierarchal clustering of miRNAs from all treatment groups. Heat map and unsupervised hierarchal clustering of sample and miRNAs were performed on the listed

samples using the 50 microRNAs that have the largest coefficient of variation based on TMM counts. Each row represents one miRNA and each column represents one sample. The color represents the relative expression level of a transcript across all samples. The red represents an expression level above the mean; green color represents an expression level below the mean.

3.4.6 Association of negative energy balance on EV-coupled miRNAs expression in blood serum

Comparative analysis of differential expression between the EV-coupled miRNAs of the ANCs and APCs lactating cow at weeks 5 and 6 of postpartum revealed that 6 miRNAs including bta-miR-122, bta-miR-375, bta-miR-145 were downregulated and the other including bta-miR-133a bta-miR-9-5p and bta-miR-184 were found to be upregulated in ANCs. Furthermore, comparison of these ANCs at week 5-6 vs heifers showed the major downregulation of differentially expressed EV-coupled miRNA in ANCs (20 out of 32) miRNAs in the ANCs as shown in table 2.

Table 3.2: List of differentially expressed miRNAs in always negative cows compared to heifers

miRNAs Name	Always negative TMM	Heifers TMM	Log2 Fold change	P-value	miRNAs name	Always negative TMM	Heifers TMM	Log2 Fold change	P-value
bta-miR-455-5p	0	87.79	-5.60747	0.0036	bta-miR-378	403.44	911.79	-1.17439	0.0122
bta-miR-214	81	555.24	-2.75716	0.0001	bta-miR-224	249.97	546.65	-1.12857	0.0236
bta-miR-122	2462.05	14519.69	-2.55716	0.0000	bta-miR-320a	2835.94	5763.54	-1.02289	0.0111
bta-miR-1246	744.87	3996.76	-2.42096	0.0285	bta-miR-150	3178.04	6440.57	-1.01666	0.0432
bta-miR-375	233.83	1195.58	-2.3381	0.0000	bta-miR-1306	694.51	336.33	1.00064	0.0322
bta-miR-199c	52.38	255.47	-2.29076	0.0017	bta-miR-374a	623.88	293.23	1.09156	0.0199
bta-miR-145	107.79	436.23	-2.02577	0.0005	bta-miR-10a	3170.42	1464.66	1.11496	0.0207
bta-miR-141	51.46	207.9	-2.00212	0.0053	bta-miR-30d	14515.27	6516.81	1.15255	0.0045
bta-miR-194	541.96	2008.51	-1.88682	0.0001	bta-miR-204	295.93	115.66	1.32187	0.0206
bta-miR-215	1144.56	3882.27	-1.76362	0.0001	bta-miR-197	299.32	114.9	1.328	0.0247
bta-miR-130a	86.78	287.08	-1.70881	0.0081	bta-miR-652	256.67	92.49	1.50607	0.0162
bta-miR-192	3206.73	10307.4	-1.6851	0.0000	bta-miR-2448-3p	160.17	46.24	1.79892	0.0224
bta-miR-107	687.18	2136.34	-1.63264	0.0001	bta-miR-2285p	123.93	25.02	2.11883	0.0112

bta-miR-140	1029.07	3152.57	-1.60759	0.0008	bta-miR-362-5p	84.03	10.64	2.76433	0.0085
bta-miR-143	4110.74	12497.15	-1.60534	0.0011	bta-miR-184	1788.92	77.8	4.53191	0.0011
bta-miR-199a-3p	10814.12	27264.23	-1.33381	0.0244	bta-miR-9-5p	1066.6	31.93	4.96909	0.0002

Extended list of miRNAs with the corresponding raw read count and FDRs are presented in Supplementary Table S 1

Moreover, we analyzed the differential expression of miRNAs in the TCs at two different time windows. Expression analysis of EV-coupled miRNAs in TCs at week 5-6 compared to heifers revealed that 41 differentially expressed miRNA and out of these 28 miRNAs were downregulated and only 13 miRNAs were upregulated in TCs as shown (Table-3).

Table 3.3: Differentially expressed miRNAs in transient cows at weeks 5 & 6 and heifers

miRNAs name	Transient cows TMM	Heifers TMM	Log_2 Fold changes	P-value	miRNAs name	Transient cows TMM	Heifers TMM	Log_2 Fold changes	P-value
bta-miR-206	93.02	3794.38	-5.35412	0.0000	bta-miR-199a-3p	6982.97	26415.07	-1.91836	0.0000
bta-miR-133a	43.98	1081.26	-4.68326	0.0000	bta-miR-2284w	50.88	173.33	-1.65296	0.0031
bta-miR-215	167.2	3761.91	-4.54603	0.0000	bta-miR-140	968.47	3054.47	-1.64392	0.0000
bta-miR-122	747.12	14068.48	-4.22894	0.0000	bta-miR-199a-5p	72.66	212.91	-1.45116	0.0101
bta-miR-375	71.44	1158.44	-4.12767	0.0000	bta-miR-152	761.06	1973.52	-1.37539	0.0001
bta-miR-1	136.29	2292.42	-4.12449	0.0003	bta-miR-320a	2172.51	5584.6	-1.35952	0.0000
bta-miR-214	30.91	537.96	-3.94985	0.0000	bta-miR-432	86.84	225.32	-1.32715	0.0135
bta-miR-378c	6.59	79.48	-3.63642	0.0020	bta-miR-204	237.65	112.08	1.03822	0.0417
bta-miR-199c	27.46	247.54	-3.21138	0.0000	bta-miR-374b	345.26	160.57	1.04547	0.0133
bta-miR-192	1084.91	9988	-3.20598	0.0000	bta-miR-421	486.35	229.56	1.07182	0.0075
bta-miR-1246	526.27	3871.8	-2.88255	0.0008	bta-miR-1468	4403.55	2099.53	1.07526	0.0002
bta-miR-143	1864.95	12110.07	-2.70324	0.0000	bta-miR-30d	13603.08	6315.16	1.10463	0.0012
bta-miR-193a-5p	37.19	246.12	-2.63472	0.0000	bta-miR-30b-5p	506.98	227.23	1.11121	0.0211
bta-miR-130a	47.43	278.16	-2.54747	0.0000	bta-miR-190a	308.17	134.42	1.23623	0.0087
bta-miR-107	374.24	2070.05	-2.4585	0.0000	bta-miR-95	177.04	56.47	1.46883	0.0076
bta-miR-145	73.37	422.72	-2.44903	0.0000	bta-miR-338	289.42	90.72	1.63218	0.0007
bta-miR-194	365.03	1946.18	-2.41155	0.0000	bta-miR-652	308.09	89.61	1.81262	0.0006
bta-miR-378	171.46	883.47	-2.35854	0.0000	bta-miR-200c	566.53	101.58	2.40995	0.0124
bta-miR-382	35.27	206.53	-2.27951	0.0001	bta-miR-2336	47.32	6.69	2.64842	0.0320
bta-miR-455-5p	20.17	85.07	-2.27926	0.0219	bta-miR-628	61.11	6.94	3.16643	0.0147
bta-miR-147	14.29	69.54	-1.96006	0.0247					

Extended list of miRNAs with the corresponding raw read count and FDRs are presented in Supplementary Table S 2

Furthermore, we have performed a pairwise comparison between the ANCs vs heifers and in the TCs at weeks 5 & 6 vs heifers. Interestingly, 17 miRNAs were downregulated and three

miRNAs were upregulated in both ANCs and TCs compared to heifers as indicated in Fig. 3.5.

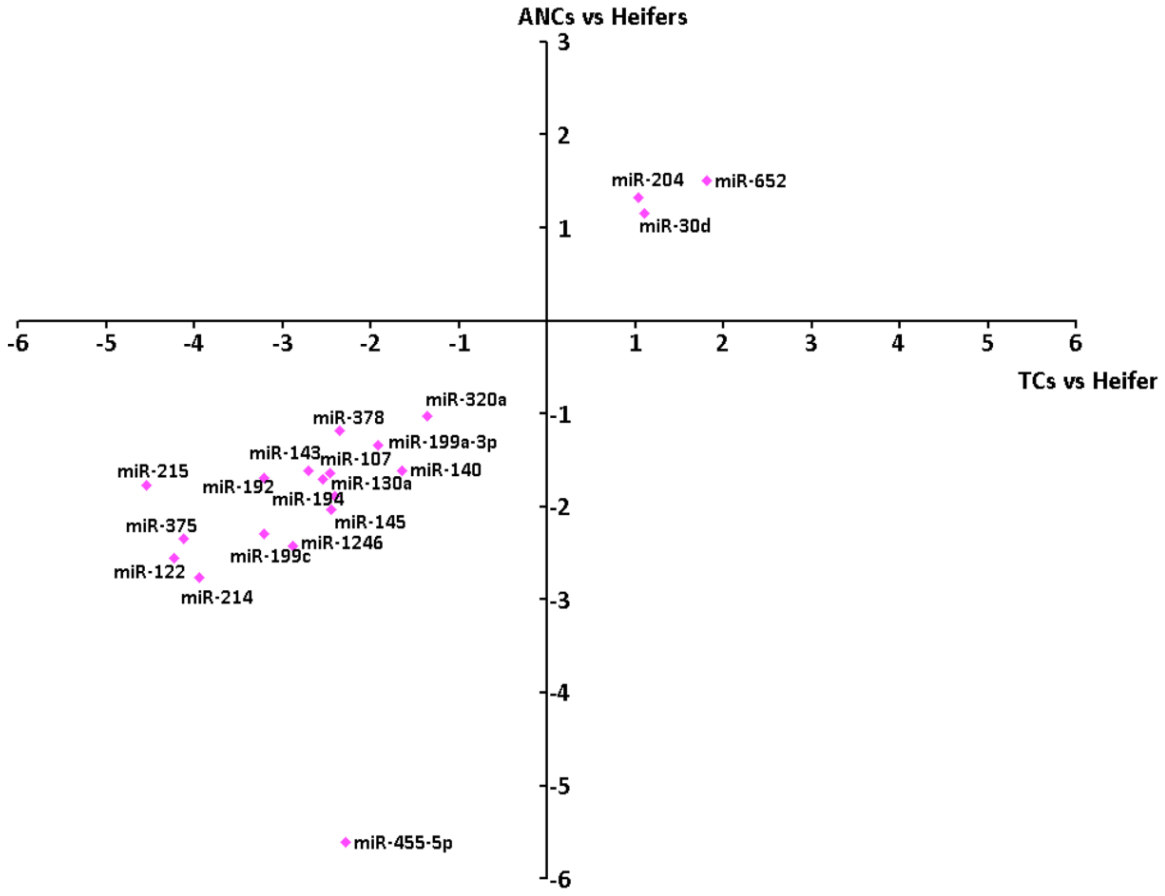
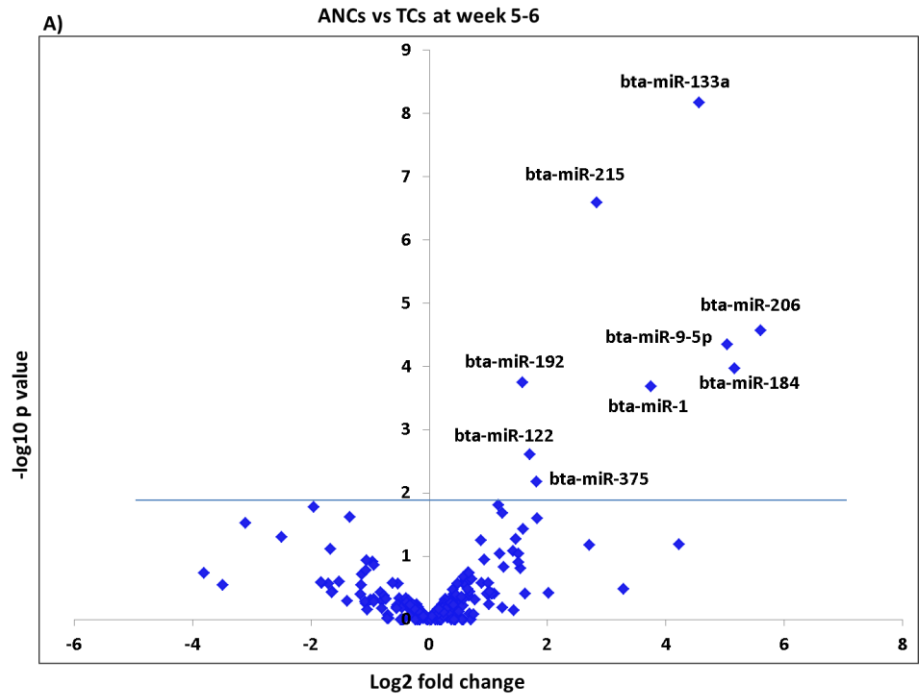


Figure 3.5: Commonly expressed EVs-coupled miRNA in both pairwise comparisons between the ANCs vs heifer and the TCs vs heifer**.** The X-axis indicates the $\log_2$ fold change of TCs vs heifers and the Y-axis indicates the $\log_2$ fold change of ANCs vs heifers.

3.4.7 Identification of EV-coupled miRNAs in blood serum as an indicator of recovery from metabolic stress

For this, we performed a comparison of EV-coupled miRNAs abundance in APCs vs TCs at weeks 5 and 6. Our results revealed the upregulation of the all 9 differentially expressed miRNA in ANCs as indicated in Fig. 6A. Furthermore, the comparison of TCs at week 5-6 compared to their week 9-10 of the same animal revealed downregulation of all differentially

expressed miRNAs (Fig. 6B). From these miRNAs, five miRNAs; miR-133a, bta-miR-215, bta-miR-206, bta-miR-192, bta-miR-122, and bta-miR-375 were upregulated in ANCs compared to TCs at week 5-6. These, miRNAs were downregulated in the comparison of TCs at week 5-6 and week 9-10.



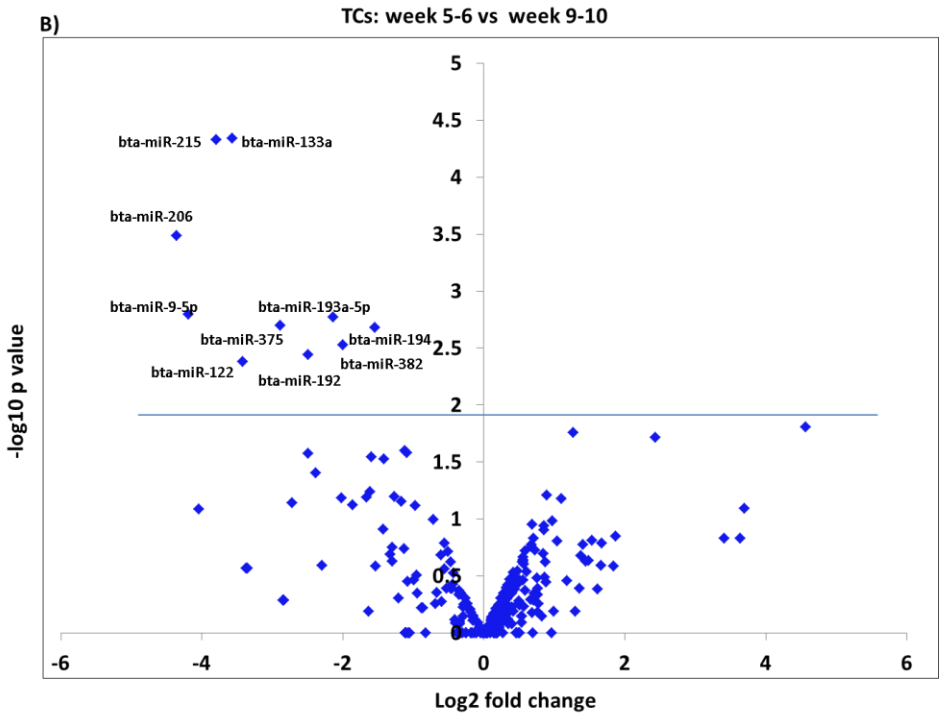


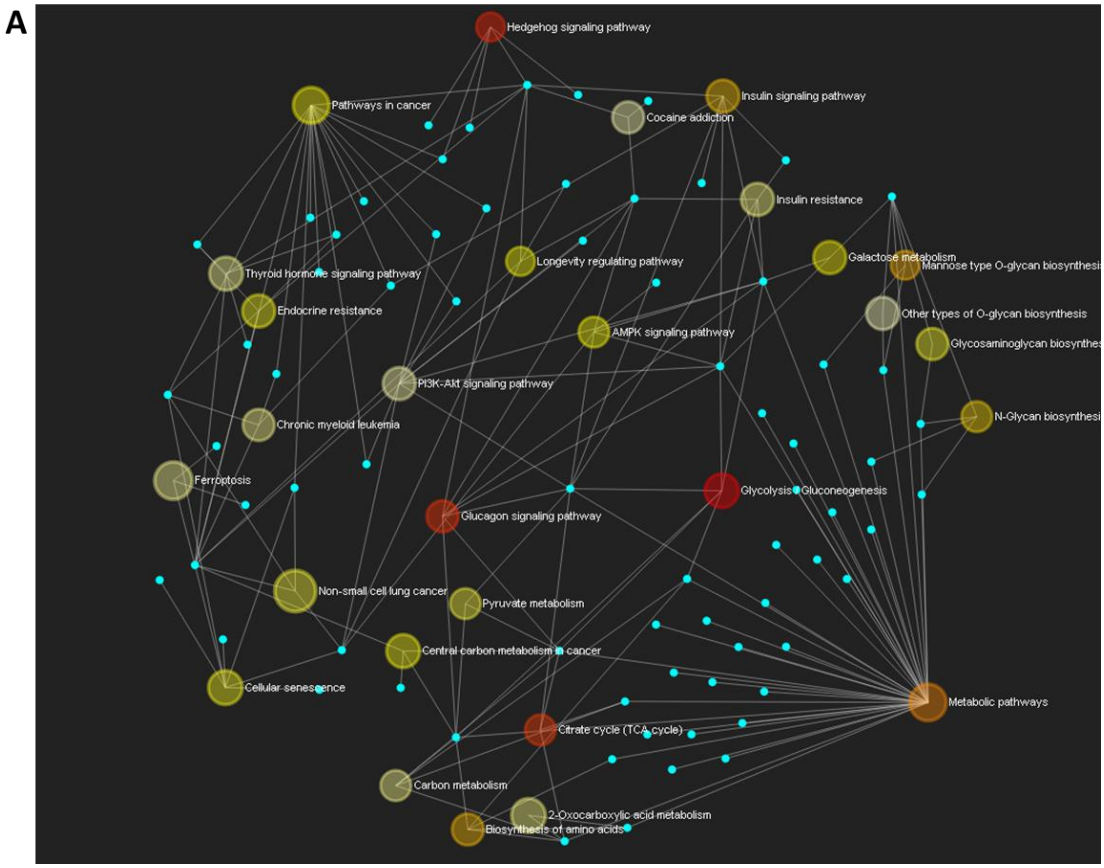
Figure 3.6: Volcano plot of differential expressed miRNAs comparisons. A) APCs compared to TCs at week 5-6. B) TCs at week 5-6 compared to week 9-10. The P-value was transformed to -log10 and above the blue line indicates significantly expressed known miRNAs.

3.4.8 Effect of lactation on the expression of EV-coupled miRNAs in blood serum

To investigate the effect of lactation on the dynamics of EV-coupled miRNAs in the blood serum, we have compared APCs verses heifer. Both groups of cows have a positive energy balance and the lactation physiology is different in these groups. The result revealed on the investigating lactation induced EV-coupled miRNAs was performed in the blood serum of the APCs compared to heifers. Our result revealed that only two miRNAs: bta-miR-206 and bta-miR-133a were differentially expressed and both of them were downregulated in always positive cows or lactating cows.

3.4.9 Target prediction of EV-coupled miRNAs in blood serum of metabolically divergent cows'

To investigate the function of differentially expressed miRNAs we have performed target prediction of miRNAs for the top five downregulated during metabolic stress being commons to the comparison of ANCs vs heifers and TCs vs heifers at week 5-6. For this, the target genes of the top five EV-coupled miRNAs; bta-miR-215, bta-miR-122 bta-miR-375, bta-miR-214, and bta-miR-199c were uploaded into network analysist software. Their target genes were found to be involved in different pathways related to metabolism (Fig. 3.8A). Moreover, target prediction analysis for the commonly upregulated EV-coupled miRNAs; bta-miR-204, bta-miR-30d, and bta-miR-652 was performed (Fig. 3.8B).


A
Hedgehog signaling pathway
Pathways in cancer
Insulin signaling pathway
Cocaine addiction
Insulin resistance
Longevity regulating pathway
Galactose metabolism
Mannose type O-glycan biosynthesis
Thyroid hormone signaling pathway
Endocrine resistance
Other types of O-glycan biosynthesis
AMPK signaling pathway
Glycosaminoglycan biosynthesis
PI3K-Akt signaling pathway
Chronic myeloid leukemia
N-Glycan biosynthesis
Ferroptosis
Glycolysis
Gluconeogenesis
Glucagon signaling pathway
Non-small cell lung cancer
Pyruvate metabolism
Central carbon metabolism in cancer
Cellular senescence
Metabolic pathways
Citrate cycle (TCA cycle)
Carbon metabolism
2-Oxocarboxylic acid metabolism
Biosynthesis of amino acids

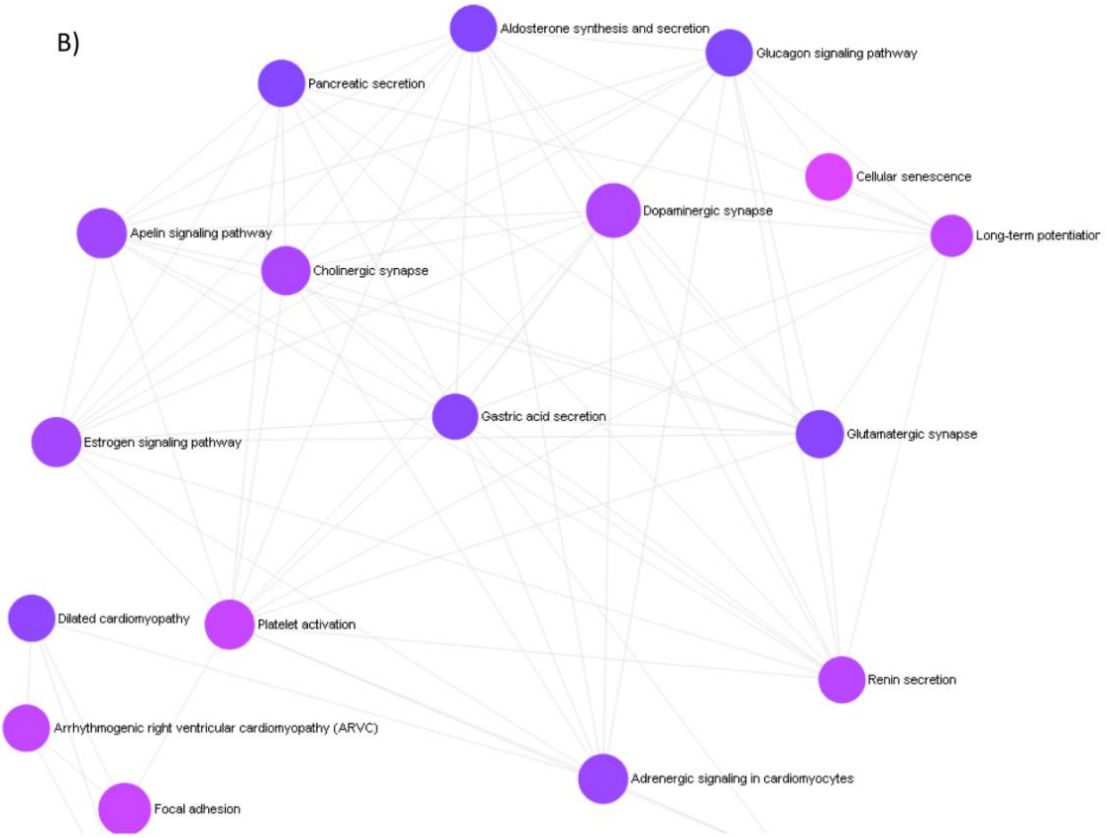

B)
Aldosterone synthesis and secretion
Glucagon signaling pathway
Pancreatic secretion
Cellular senescence
Dopaminergic synapse
Apelin signaling pathway
Long-term potentiation
Cholinergic synapse
Gastric acid secretion
Estrogen signaling pathway
Glutamatergic synapse
Dilated cardiomyopathy
Platelet activation
Renin secretion
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Adrenergic signaling in cardiomyocytes
Focal adhesion

Figure 3.7: Pathways regulated by differentially expressed miRNAs in ANCs vs heifers and TCs vs heifers and pathway analysis. A) Pathways regulated by all downregulated miRNAs, B) pathways regulated by upregulated miRNAs

3.5 Discussion

In this study, we have analyzed the correlation between the postpartum negative energy balance and the dynamic change in EV-coupled miRNAs in the blood serum of divergent metabolic stats postpartum lactating cows and heifers. During the last few decades, more comprehensive genetic selection strategies have been developed and implemented to improve reproductive performance while maintaining high milk production in dairy cattle. A previous study reported that severe negative energy balance on bovine hepatic tissue during negative energy balance was reported to downregulate the majority of genes in which out of 416 genes, about 227 were reported to be downregulated by negative energy balance in high-yielding dairy cows during day-14 of the postpartum period (McCarthy *et al.* 2010). Several studies evidenced that, NEB is associated with the dynamic change of the reproductive hormone. Previously, early postpartum dairy cows have been documented to have very low progesterone (Savio *et al.* 1990), low blood plasma FSH (Opsomer *et al.* 1996), and low oestradiol concentrations (Dumesic *et al.* 2015). Here the association between the dynamic changes of EV-coupled miRNAs shuttle molecules in the blood serum as a noninvasive indicator of metabolic stress in dairy cows during early postpartum has analyzed.

In this study, the dynamic change of EV-coupled miRNAs shuttle molecules in the blood serum as a noninvasive indicator of metabolic stress in dairy cows during their early postpartum have been investigated. Advanced reproductive technology has revolutionized the dairy industry since the invention of artificial insemination first successfully applied in cattle in the 1900s followed by the next major developments involved semen extenders, an

invention of the electro-ejaculator, progeny testing, sperm cryopreservation with glycerol (Moore & Hasler 2017). These huge increments in milk production capability in dairy cows have documented to be associated with declining in fertility due to higher energy expenditure. Previous studies have shown that increased NEFA and BHB concentration in the bloodstream of metabolically stressed cows resulted in disturbed reproductive function (Leroy *et al.* 2008b). Therefore, identifying metabolic-related dynamic change in the blood serum could give insight into identifying specific miRNA molecules associated with lactating cows' metabolic stress.

In this experiment, the analysis of postpartum lactating cow's blood serum metabolite revealed that three different types of energy status (Table 3.1). The majority of them were TCs to the onset of NEB with a high concentration of both NEFA (0.82±0.32 mmol/L) and ß-hydroxybutyrate (0.85±0.36 mmol/L) at week 5 of postpartum. Starting, week 7 and 8, these type of cows were able to recover from the negative energy and the concentration of these metabolites was decreased continuously in their blood serum as low as NEFA to 0.41± 0.22 mmol/L and ß-OHB to 0.52±0.10 mmol/L at week 10 shows recovery from NEB. The second group of cows were found to be ANCs and having a high concentration of NEFA with a range of 0.69±0.08 to 1.05±0.06 mmol/L through the study period. Similarly, their ß-hydroxybutyrate concentration level throughout their early lactation was constantly high concentration with a range of 0.78± 0.17 to 0.98±0.02 mmol/L during the study period showing clinically at NEB.

The third type of cows was found to be APCs in which the concentration of blood serum metabolites was lower throughout the early lactation and study period. These types of cows were having a lower NEFA concentration of 0.40±0.10 to 0.26±0.19 mmol/L at with a consistent across the experimental weeks. Similarly, the concentration of ß-hydroxybutyrate was as low as 0.53 ±0.15- 0.42 ±0.17 mmol/L during the study period. This indicates that the

absence of clinical NEB in these cows during their early lactation. In case of heifers, we have found very low concentration of NEFA concentration 0.19± 0.13 and 0.07±0.03 mmol/L in circulation during the study period. Similarly, we have found a low concentration of ß-hydroxybutyrate ranged 0.38±0.14 and 0.46±0.05 mmol/L throughout the study weeks. Previously, the rapid body weight loss and body condition score loss are the most obvious clinical symptoms of forced lipid mobilization due to NEB in early lactation (Huszenicza *et al.* 2004). These results revealed individual differences between cows in terms of dealing with metabolic stress during early lactation and the summarized in (Fig. 3.1).

In this experiment, global expression analysis of EV-coupled miRNAs using next-generation sequencing in the blood serum of different metabolic status cows and heifers was performed. We have detected a large number of known EV-coupled miRNAs across all samples and most of them were commonly expressed (Fig. 3.2) showed a housekeeping role. Previously, circulatory miRNAs are optimal biomarkers owing to high stability under storage and handling conditions and their presence in blood and other biological fluids. Interestingly, we have observed the change in the dynamics of several uniquely expressed miRNAs as the metabolic status changed. The most uniquely expressed EV-coupled miRNAs in the blood serum were detected in the APCs cows and among them, bta-miR-505, bta-miR-452, and bta-miR-6119-3p are x-linked uniquely expressed miRNAs. The other two bta-miR-29d-5p and bta-miR-429 located in chr16 were also uniquely expressed in APCs suggested lactation induced miRNAs. Previous study showed that high lactation performance was associated with a high abundance of miRNA-29 expression in dairy cow mammary epithelial cells (Melnik 2015). Moreover, we have observed several uniquely expressed miRNAs in ANCs including bta-miR-2299-3p, bta-miR-362-5p, and bta-miR-22-5p and could be associated to lower feed conversion efficiency and immune response which leads these cows to stay at

metabolic stress for long term. Previous study reported that lower expression rumen miR-2299-3p is positively associated with N efficiency (Wang *et al.* 2016).

In this study, we have shown the association of negative energy balance in early lactating cows and the relative abundance of EV-coupled miRNAs in blood serum of metabolically stressed cows both the ANCs and TCs were compared using hierarchical clustering. Hierarchical clustering of differential expressed miRNAs showed that, downregulation of EV–coupled miRNAs in the replicates of ANCs, TCs and upregulation of those clustering miRNAs in APCs and heifer (Fig. 3.3). This suggests metabolic stress during lactation could suppress the release of EV-coupled miRNAs in the circulation. Previous study showed that severe negative energy balance to be associated with the alteration of hepatic miRNA expression (Fatima *et al.* 2014c), hepatic gene expression (McCarthy *et al.* 2010) and reduced IGFBP-2 and IGFBP-6 gene expression in the oviduct tissue in dairy cows (Fenwick *et al.* 2008).

In this study, pairwise differential expression analysis on metabolically stressed and non-stressed cows revealed that suppression on the majority of differential expressed EVs coupled miRNAs due to metabolic stress during their early lactation. Differential expression analysis on ANCs vs heifers revealed that massive downregulation (~63%) of EV-coupled miRNAs in ANCs at week 5-6 compared to heifers (Table 2). Indeed, suppression on the majority of EV-coupled miRNAs (> 68 % of them) in the TCs at week 5-6 compared to heifers was also observed (Table 3). This suggests negative energy balance in lactating cows could be associated with major suppression of EV-coupled miRNAs in blood serum. This suggests metabolic stress during early lactation is associated with suppression of EV-coupled miRNAs in blood serum. Surprisingly, 19 miRNAs were found to be commonly differentially expressed with a similar patterns in both comparisons (16 upregulated and 3 miRNAs

upregulated) in both comparisons (Fig. 3.4). This suggests the association of NEB with suppression of EV-coupled miRNAs in the blood serum of metabolically stressed

In this experiment, we have analyzed the association of EVs-coupled miRNAs abundance and recovery of metabolic stress during the early and late lactation in blood serum. Differential expression analysis of EV-coupled miRNAs in TCs during their metabolic stress at week 5-6 compared to their recovery time at week 9-10 revealed that downregulation of all differentially expressed miRNAs at their week 5-6 and upregulated during their week 9-10 as indicated in Fig. 3.5A. Furthermore, we have performed the comparison of EV-coupled miRNAs abundance in APCs vs TCs at weeks 5 and 6 and revealed upregulation of the all differentially expressed miRNA except one miRNA was found to be downregulated in the APCs as indicated in fig. 3.5B. Interestingly, five miRNAs commonly differentially expressed miRNAs; bta-miR-122, bta-miR-215, bta-miR-375, bta-miR-193a-5p, and bta-miR-194 were found to be upregulated in APCs vs TCs at week 5-6 and those miRNAs were found to be downregulated in TCs at week 5-6 vs TCs at week 9-10. This suggests upregulation of those miRNAs could explain the recovery of those cows from metabolic stress.

In our analysis, we have also performed the comparison of APCs vs heifers to indicate if lactation induced EV-coupled miRNAs abundance in blood serum. Our result revealed that we did not found lactation induced EV-coupled miRNAs except downregulation of two muscle-specific miRNAs: bta-miR-206 and bta-miR-133a in blood serum. Thus, the downregulation of those miRNAs in the APCs compared to heifers. Previously, miR-206 was reported to promote the differentiation of myoblasts and miR-133 has reported promoting cell proliferation (Zhang *et al.* 2016). Furthermore, this suggests there is less chance of changing on EVs due to the milk miRNAs are expressed in the mammary gland. Previous study on comparative analysis of miRNA distribution in milk and blood serum showed that milk

miRNAs are primarily expressed from the mammary gland rather than transferred from maternal circulating blood (Modepalli *et al.* 2014). Target prediction analysis of EV-coupled specific miRNAs; bta-miR-215 and the liver-specific miRNA; bta-miR-122 has revealed to involve involvement in different metabolic-related pathways including gluconeogenesis, hedgehog signaling pathway, glucagon signaling pathway, citrate cycle, metabolic , biosynthesis of amino acids, insulin signaling pathway, galactose metabolism, longevity regulating , insulin resistance, pyruvate metabolism and carbon metabolism. This suggests their potential involvement of the target gene of those miRNAs in metabolism and reproduction could partially explain the metabolic stress of those cows.

In conclusion, EV-coupled miRNAs hierarchical and differential expression is most likely to be associated with suppression of miRNA. This study could give information as a potentially noninvasive indicator of metabolic stress in high yielding cows during their early lactation.

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Chapter 4: General discussion and conclusions

4.1 General discussion and conclusions

This PhD study was conducted to analyze the association between the physiology of negative energy balance (NEB) in high producing dairy cows and the dynamic change of extracellular vesicle-coupled miRNAs in follicular fluid and the blood serum during early weeks of post-calving. For more than a century, assisted reproductive technology especially artificial insemination had magnificently revolutionized the dairy industry. This huge increment in milk production capability has been associated with high energy expenditure in dairy cows. As a consequence, during early lactation animals enter a state of negative energy balance which resulted in the disturbing follicular microenvironment (Leroy *et al.* 2008b). In this experiment, the metabolic status of the individual cows was determined based on blood metabolite analysis, body weight, and overall energy balance based on dry matter intake and milk production measurements during the early weeks (5-10) of post-calving. Therefore, based on these metabolic parameters, animals were classified within the status of NEB or not, and follicular fluid or blood serum samples were pooled for each experimental group at week 5 and 6 and at week 9 and 10 to have best the coverage of NEB physiology during early lactation and to increase the number of animals per group.

Advancements in next-generation sequencing (NGS) technologies over the last few years allow us to sequence the genome of various species with minimum input of total RNA. Next-generation sequencing technology has been documented to pave the way to understand how organisms' genome activity is associated with their survival, reproduction, disease resistance genetic diversity and environmental interaction (Jerzy 2016). Identification of differential genes expression pattern between specific conditions is key to understand the molecular background of specific phenotypic variations (Costa-Silva *et al.* 2017). In this study, NGS was employed as a method to profile the dynamic expression EV-coupled miRNAs contents

released into follicular fluid microenvironment and in the blood serum in postpartum dairy cows and heifers.

Blood serum metabolite analysis of NEFA and BHO in postpartum cows was performed to determine the metabolic status of postpartum cows and heifers (Chapter 2 Fig. 2.1). It is well established that, a higher concentration of NEFA and BHO in the blood of dairy animals is an indicator of clinical NEB. In the present study, cows were considered to be in NEB based on the threshold for NEFA and BHO serum concentrations determined previously as >0.55 mmol/L (Fenwick *et al.* 2008) for NEFA and >0.65 mmol/L (Girard *et al.* 2015) to BHO. Accordingly, two-thirds (66%) of the cows were having transient energy balance in which, they were under NEB at early time points (week 5-6) followed by a continuous recovery during week 7-8 and full recovery at week 9-10 (Chapter 2 Fig. 2.1). Some portion of cows (17%) were continuously under NEB during the study period while others (17%) were at always positive energy balance during the period (Chapter 2 Fig. 2.1). The differences in the length of time for recovery between the ANCs and TCs could be associated with individual variations to the onset NEB and/ the difference in dry matter intake.

Previously, variation between cows in the state of entering to NEB has been reported to be directly related to differences in dry matter intake during the transition period of peripartum or postpartum (Butler 2003), therefore this could explain the fact that some cows were at NEB during several months of postpartum. A high concentration of circulating NEFA was observed in 67% of the cows (NEFA >400 µmol per liter) during their first month of lactation of which 7% those cows were reported to have high concentration until mid-postpartum at 3 to 5 months of lactation (Gonzalez *et al.* 2011). However, in the present study, the percentage of animals that remained at NEB was higher during the first 10 weeks (≈2.5 months) of post-calving compared to the previous report. This may be attributed to the higher (83%) number

of cows entered the state of NEB in this study (TCs and ANCs) compared to the previous studies during the first months. Moreover, a comparative study in 1333 cows among dry, early lactating, mid lactating and late lactating cows has reported a BHO concentration of 460 µmol/L, 567 µmol/L, 529 µmol/L and 564 µmol/L respectively in the circulation (Duffield *et al.* 1997). This supports the present study in which cows at NEB (ANCs and TCs) during early lactation were found to have high concentration BHO (0.78-0.850 mmol/L|) whereas the low concentration of BHO in case of APCs (0.53 mmol/ L) and as low as 0.38 mmol/ L for the heifers suggesting at positive energy balance.

In order to fully characterize the cows' energy status, body weight measurements were performed as supportive information to the metabolite profiling of the experimental cows (Chapter 2 Supplementary Fig. 1). Moreover, a representative body weight curve of individual cows is shown in Chapter 3 Supplementary Fig. 1. The body weight curve showed that the majority of cows loss weight as early as week 2-3 of post-calving suggesting higher lipid mobilization. Rapid body weight loss and body condition score loss were reported previously as clinical symptoms of forced lipid mobilization due to NEB in early lactation (Huszenicza *et al.* 2004). Always negative cows which had high blood serum metabolites of NEFA and BOH were found to lose their body weight until week 15^{th} of post-calving, suggesting continuous lipid mobilization throughout the experimental time. Interestingly, the APCs which had low NEFA and BOH was gaining weight which may suggest low lipid mobilization. These results were supported by an individual cow's overall energy balance (Chapter 2 Supplementary Fig. 2.2). Therefore, a model of energy balance status for metabolically divergent lactating cows during early post-calving was developed based on the above three criteria as indicated in (Chapter 2 Fig. 2.2).

After screening the energy status of experimental animals, the abundance of EVs in the FF and blood serum were investigated. It is well documented that, during folliculogenesis, there

is a secretion of different molecules to the follicular fluid which postulates to communicate either within or between the follicular cells and the oocyte. Follicular fluid is known to be enriched with numerous proteins and nucleic acids, and supplementation EVs recovered from small follicle during oocyte maturation resulted in improved cumulus cells expansion in bovine and mice with significant incensement on the relative abundance of genes (Ptgs2, Ptx3, and Tnfaip6) (Hung *et al.* 2015) and most probably released by the follicular cells or coming from the bloodstream.

Recently, studies evidenced that circulatory miRNAs molecules in the follicular fluid are carried by EVs in bovine (Sohel *et al.* 2013a), human (Santonocito *et al.* 2014) and equine (da Silveira *et al.* 2012). The number of EVs secreted to the follicular fluid was higher in small follicles compared to medium and large follicles in bovine (Navakanitworakul *et al.* 2016). The study also revealed a significantly high number of EVs to be abundant in the follicular fluid of large size follicle aspirated using OPU (Chapter 2 Fig. 2.3B). Despite the hypothesis that cell-cell communication within the follicle to be mediated by secretion and uptake of exosomes that contain several bioactive molecules including extra-cellular miRNAs (Sohel *et al.* 2013), the exact origin of those EVs in the follicular fluid was not determined. However, it is speculated that granulosa cells, theca cells, and cumulus cells could be the sources of the EVs circulating in the follicular microenvironment. Despite the fact that ovarian cells especially granulosa cells revealed a significant dynamics of miRNAs expression (Salilew-Wondim *et al.* 2014; Gebremedhn *et al.* 2015), the packaging and release of those miRNAs from those cells through EVs have not been ruled out.

In this study, FF and blood serum EVs' morphology and molecular analysis revealed that the isolated EVs were found to be good in terms of size, morphology, and specificity. The size of EVs recovered from the follicular fluid samples were found within a range of 124.8 -147.1 nm in diameter (Chapter 2 Fig. 2.3B). Interestingly, the size of EVs recovered from blood

serum samples using ultracentrifugation were found to be within a range of 113.4 -140.6 nm (Chapter 3 Fig. 3.2B). This shows that EVs from the blood serum is relatively smaller in size compared to EVs from the follicular fluid. This difference in size was further confirmed by the morphological analysis of those EVs using transmission electron microscopy (Chapter 3 Fig. 3.2A). This variation in size could be related to the function of those EVs in carrying various signal molecules within the FF or blood serum.

Large scale expression analysis of EV-coupled miRNAs in follicular fluid revealed a total of 356 known and 156 novel putative miRNAs across all the experimental groups (Chapter 2 Fig. 2.4). About 70% of the total detected miRNAs were found in all experimental groups suggesting their housekeeping roles in the follicular environment. The housekeeping role of several miRNAs in ovarian granulosa cells from dominant and subordinate follicles has been reported previously (Gebremedhn *et al.* 2015). Interestingly, the rest 30% of the detected EV-coupled miRNAs were uniquely expressed either in one or more groups of animals. This differential expression of EV-coupled miRNAs could partially explain the unique cell-to-cell communication and gene regulation mechanism during follicular development in metabolically divergent dairy cows. Furthermore, the highest number of miRNAs in total were detected in APCs (a total of 319 miRNA) and the lowest number of were detected in ANCs (a total of 290 miRNA). This finding suggests that a higher number of EV-coupled miRNAs were released from the healthy follicle to the follicular fluid than from follicles under metabolic stress conditions. Previously, a significantly larger number of cellular miRNAs have been observed in large atretic follicles compared to large healthy follicles in bovine (Sontakke *et al.* 2014).

Pairwise comparison of differential expression and hierarchical clustering revealed distinct patterns of expression of EVs-coupled miRNAs in follicular fluid from ANCs, TCs, and APCs compared to control heifers. That clustering analysis showed a major downregulation

of miRNAs on the metabolically stressed cows whereas the opposite was observed in the non-stressed cows (Chapter 2 Fig. 2.5). Previously, NEB was reported to suppress granulosa cells gene expression linked to the cellular organization, proliferation, fatty acids metabolism, and vitamins A and D (Girard *et al.* 2015). Specialized transcription factors are known to be activated in response to metabolic change and to induce changes in gene expression (van der Knaap & Verrijzer 2016). Early postpartum dairy cows have been documented to have very low oestradiol concentrations (Dumesic *et al.* 2015). Hormonal regulation of miRNAs expression is well known to be regulated by the circulating estrogen concentration (Katchy & Williams 2016). In the present study, the global suppression of EV-coupled miRNA could be associated with low levels of oestradiol in follicular fluid. This may suggest that metabolic status coupled with hormonal imbalance could affect cellular miRNAs expression and its subsequent release into the extracellular environment. A previous study revealed that in vitro supplementation of elevated NEFA during oocyte maturation altered the gene expression level of DNMT3A, IGF2R, and SLC2A1 on day 7 blastocysts (van Hoeck *et al.* 2011). Moreover, injection of NEFA (oleic acid) to the follicular fluid of the growing dominant follicles in vivo was reported to reduce intrafollicular 17β-Estradiol concentrations and altered the abundance of gene expression in granulosa cells recovered from antrum follicles' (Sharma *et al.* 2019).

In the present study, pairwise comparison of differentially expressed EV-coupled miRNAs between metabolically stressed and nonstressed cows revealed downregulation of all differentially expressed miRNAs including bta-miR-486 and bta-miR-451 (Chapter 2 Fig. 2.6). Previously, bta-miR-486 was reported to regulate lactation by targeting PTEN in mammary glands (Li *et al.* 2015) and immune regulatory mechanisms in cows (Jin *et al.* 2014). Moreover, miR-451 was reported to involve in switching between proliferation and migration by targeting AMPK signaling, mTOR modulation and Rac1 activation required in

malignant glioma cells (Zhao *et al.* 2017). The target prediction of those miRNAs revealed their involvement in various pathways associated with ovarian function including apoptosis, TGF-beta signaling, hippo signaling, lysine degradation, cell cycle, FoxO signaling, mTOR signaling, VEGF signaling and others (Chapter 2 Fig. 2.6). TGF-β signaling pathway has been reported to play a vital role in the mammalian ovary during follicle development and ovulation and its complete knockout causes embryonic lethality in mice (Yu *et al.* 2016).

Furthermore, the downregulation of the majority of EV-coupled miRNAs (25 out of 37 miRNAs) was observed in the follicular fluid of ANCs at week 5-6 compared to heifers including bta-miR-122, bta-miR-147, bta-miR-21-3p, bta-miR-150, bta-miR-146a and bta-miR-138 (Chapter 2 Table 1). Previously, increased the expression level of liver tissue miRNA; bta-miR-122 was reported to be correlated with metabolic stress in postpartum dairy cows (Fatima *et al.* 2014a). Moreover, upregulation of circulatory miRNAs in milk including bta-miR-21, bta-miR-146a and bta-miR-155 were reported to be associated with lactating cows affected by mastitis (Lai *et al.* 2017) suggesting the involvement of those miRNA in immunological activities. The target prediction of those differentially expressed and downregulated miRNAs was found to involve in different pathways (Chapter 2 Supplementary fig. 2.S4). Those predicted targeted have previously documented to be involved in follicular cell's proliferation and oocyte developmental competence (Progesterone-mediated oocyte maturation, oocyte meiosis, estrogen signaling pathway), in metabolism (fatty acid elongation, fatty acid metabolism, non-alcoholic fatty liver disease, fatty acid biosynthesis, vitamin B6 metabolism, insulin signaling, AMPK signaling), in immunological (PI3K-Akt signaling, TNF signaling, mTOR signaling, and regulation of actin cytoskeleton) and other signaling pathways (MAPK signaling, focal adhesion).

In the current study, in addition to the follicular fluid, holistic approach expression analysis of EV-coupled miRNAs was investigated in blood serum from the TCs at week 5-6 and week 9-

10, ANCs at week 5-6, APCs at week 5-6 and heifers at the same time point as follicular fluid (Chapter 3 Fig. 3.1). Thus, EV-coupled miRNAs detection analysis using NGS in blood serum revealed that a total of 179 previously known and 118 novel miRNAs were found across all metabolic status cows (Chapter 3 Fig. 3.3). The number of housekeeping EV-coupled miRNAs in blood serum was about 81% and the number of uniquely expressed miRNA accounted for 20% (Chapter 3 Fig. 3.3). The number of totals detected known and uniquely EV-coupled miRNAs across all metabolic status cows and heifers were lower than the follicular fluid.

In order to associate the energy status and the abundance of the EV-coupled miRNAs, hierarchical clustering of differentially expressed miRNA was performed in the blood serum of all replicates of different metabolic status cows as indicated in chapter 3 Fig. 3.2. As it is observed in FF, EV-coupled miRNA expression in blood serum is also associated with the metabolic status of the animals. Furthermore, pairwise comparison of EV-coupled miRNAs in the ANCs vs heifers revealed the massive downregulation of EV-coupled miRNAs (20 out of 32 miRNAs) in ANCs (Chapter 3 Table 1). Interestingly, the comparison of TCs vs heifers has revealed a similar pattern of miRNA expression in which 28 out of 41 DE miRNAs were downregulated in TCs at week 5-6 (Chapter 3 Table 2). In both comparisons, 20 miRNAs were found to be common differentially expressed with similar patterns of expression (17 down and 3 upregulated) as shown (Chapter 3 Fig 3.5). This downregulation of EV-coupled miRNAs in blood serum is associated with metabolic stress in early lactating postpartum cows and could be associated with the low estradiol concentrations in the body during NEB.

Target predicted genes of the top five commonly downregulated EV-coupled miRNAs in both (ANCs and TCs compared to heifers); bta-miR-215, bta-miR-122 bta-miR-375, bta-miR-214, and bta-miR-199c revealed significantly pathways associated with metabolism, endocrinological, lactation and immunology (Chapter 3 Fig. 3.8A). Pathways associated with

metabolism include hedgehog signaling, metabolic, carbohydrate digestion and absorption, gluconeogenesis, mineral absorption, fatty acid biosynthesis, thiamine metabolism, and glucagon signaling. Moreover, the target genes also involved in endocrinological pathways include endocrine resistance, insulin resistance, insulin secretion, thyroid hormone synthesis, and insulin signaling pathways. Furthermore, those miRNAs involved in immunological pathways include (autophagy-animal and mitophagy-animal pathways), and lactation pathways (prolactin signaling, factor-regulated calcium reabsorption andTGF-beta signaling). Previous study showed that NEB is associated with undesired complications including suppression of dry matter intake, immunosuppression, and consequent infertility during the transition period (Wankhade *et al.* 2017).

The comparative analysis of the expression pattern of EV-coupled miRNAs in FF and their corresponding blood serum of cows was performed to investigate conserved miRNAs candidates which can be well associated with NEB in dairy cows. For this, we have compared the commonly DE in ANCs vs heifers in both follicular samples and blood serum of the same cows. The results revealed that four differentially expressed miRNAs were having similar patterns in both blood serum and follicular fluid. Out of these, bta-miRNA-122 and bta-miRNAs-150 were downregulated and bta-miRNA-9-5p and bta-miRNA-196 were upregulated in both comparisons. This suggests that specific miRNAs could be shuttled from the blood serum to the follicular fluid and vise verse through extracellular vesicle. A previous study reported that downregulation of serum circulatory miR-122 was associated with uterine infection in cows compared to the normal uterus (Kasimanickam & Kastelic 2016). Furthermore, the upregulation of EV-coupled miR-150 was reported in the large follicles and supporting follicle growth and ovulation of oocytes by targeting the regulation of VEGF (Navakanitworakul *et al.* 2016b). Even though both in follicular fluid and blood serum the hierarchical clustering and pairwise comparison is a similar pattern, the number of commonly

differentially expressed miRNAs are few compared to a large number of differentially expressed EV-coupled miRNAs during early post Calving. This could be associated with the fact that the main sources of EV-coupled miRNAs in the follicular fluid are mainly from follicular cells.

Comparative analysis of ANCs vs TCS, and TCs at week 5-6 vs week 9-10 revealed those miRNAs to be associated with recovery from metabolic stress in follicular fluid. Comparative analysis of EV-coupled miRNAs in ANCs vs TCs at week 5-6 postpartum could reveal, potential miRNAs as predictors of cows that will maintain negative energy balance or those that will recover in the later stages of lactation at weeks 9-10 postpartum. Cows, which remained at NEB until week 9-10, suppressed the expression of miR-132, bta-miR-34b and bta-miR-34c and bta-miR-449a in follicular fluid. Previously, miR-132 and miR-34 were reported to be highly abundant in the follicular fluid of cows that under-went ovarian hyperstimulation compared to the unstipulated ones (Noferesti *et al.* 2015). Moreover, the comparison of differentially expressed miRNAs between EVs recovered from follicular fluid collected from transient cows at weeks 5-6 and weeks 9-10 revealed that bta-miR-34b, bta-miR-34c, and bta-miR-449a were upregulated, whereas two miRNAs, bta-miR-451 and bta-miR-592, were downregulated at weeks 5-6. The upregulation of those miRNAs in blood serum could be associated with the recovery of those cows from metabolic stress.

In this study, lactation caused the induction of EV-coupled miRNAs in follicular fluid but not in blood serum. Interestingly, comparative analysis between APCs and heifer revealed the upregulation of massive EV-coupled miRNAs in follicular fluid (Chapter 2 Table 1). Cows under positive energy balance postpartum showed induction of 31 out of 38 DE miRNAs compared to unstressed control heifers (Chapter 2 Table 2). Unlike the follicular fluid, the comparison of APCs vs heifers in blood serum showed downregulation of only bta-miR-206 and bta-miR-133a.

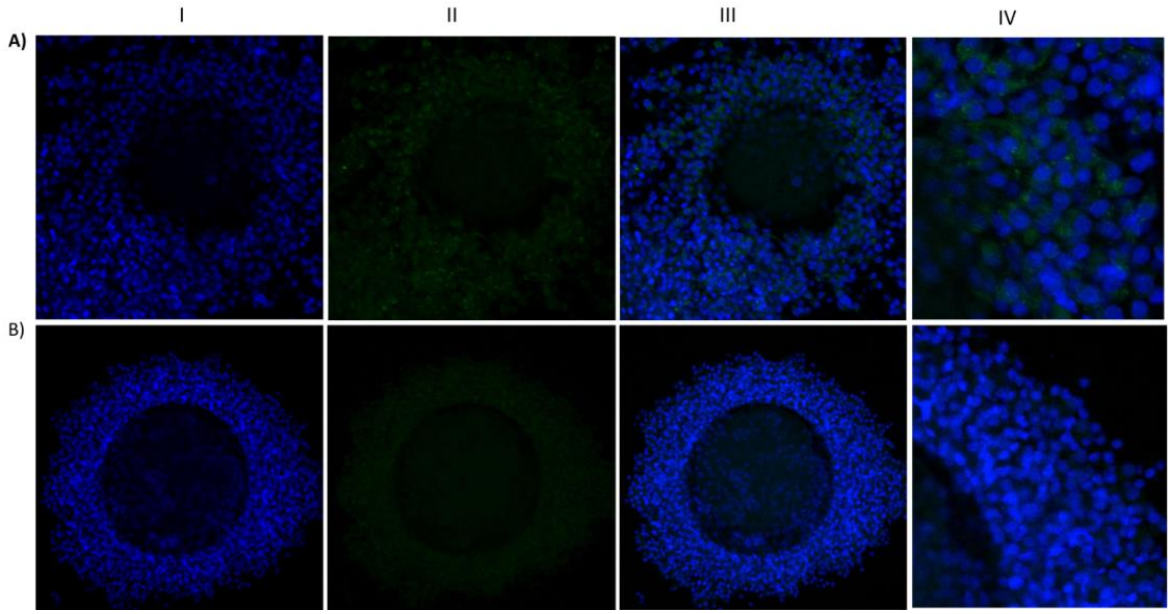

Figure 4.1: Uptakes of EVs by matured cumulus-oocyte-complex (COC) during oocyte maturation. COC were co-incubated for 22 hrs with EVs and images were taken after maturation by confocal microscopy. A) Shows COC co-incubated with labeled EVs at the immature stage. B) COC co-incubated with only labeled PBS during maturation. Images were taken after maturation under confocal microscopy II, III, IV, and I represent; only DAPI, PKH67, DAPI+PKH67 and cropped DAPI+PKH67 respectively.

The analysis of EVs in denuded oocyte shows no uptake by the oocyte under confocal microscopy (Chapter 4 Fig. 4.2). Nevertheless, more validation should be done to investigate the interaction with the zona pellucida permeability and the EVs. EVs co-incubation of EVs recovered from follicular fluid with cumulus-oocyte-complexes showed uptake of extracellular vesicles by the cumulus cells as shown in. This study revealed how EVs are uptaken by the cumulus cell but not by the oocytes. However, different mechanisms should be implemented for further validation. This was accompanied by, a relatively lower tendency of oocyte maturation rate in oocytes co-incubated with EVs recovered from follicular fluid from cows at continuous NEB compared to heifers. Taking the polar body extrudsion as parameters, the various experimental groups including those co-incubated with EVs from

ANCs, APCs, TCs (5-6 weeks), TCs (9-10 weeks) and heifers showed the 69%, 71%, 78.4%, 80.75%, and 77.4% respectively.

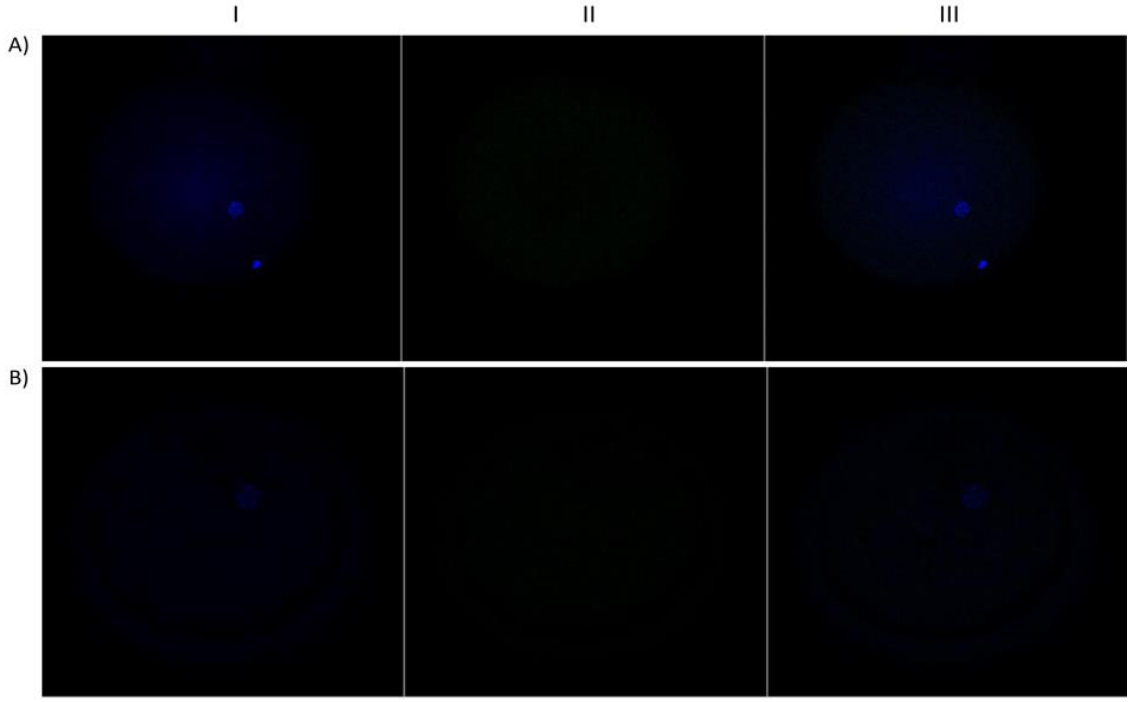

Figure 4.2: Uptakes of EVs by the oocyte after maturation and cumulus cells were removed manually after maturation. Uptakes of EVs by the oocyte after maturation and cumulus cells were removed manually after maturation. COC were co-incubated for 22 hrs with EVs and images were taken after maturation by confocal microscopy. A) Shows COC co-incubated with labeled EVs at the immature stage. B) COC co-incubated with only labeled PBS during maturation. Images were taken after maturation. I, II, and III represent; only DAPI,) PKH67 and DAPI+PKH67 respectively

4.2 Conclusions and future prospectives

In conclusion, there is a variation in the resistance to NEB among the dairy cows and 83% are either transient or continuously at metabolic stress. Nevertheless, some portion of the lactating cows is inherently resistant to metabolic stress regardless of milk production and those groups of cows could be selected for future breeding programs. In this study, NEB post

calving in early lactating cows is mainly associated with downregulation of EV-coupled miRNAs in dairy cows' in both follicular fluid and blood serum which may partially explain reduced fertility in cows due to post-calving metabolic stress. Even though negative energy balance was associated with major downregulation of EV-coupled miRNAs in both follicular fluid and blood serum, a low degree of EV-coupled miRNAs conservation was found between follicular fluid and blood serum. Moreover, during NEB, few blood serum EV-coupled miRNAs could be reflected in the follicular fluid microenvironment of the same cows and could be used as a noninvasive indicator of metabolically stressed cows. Surprisingly, lactation has an effect on EV-coupled miRNAs in follicular fluid cows compared to heifers but not in blood serum. However for the future;

- Further studies on the in vitro development, fertilizability, and blastocyst rate of the individual oocyte recovered from large follicles from the follicular fluid of different metabolic status cows could give additional information on the effect of metabolic stress on oocyte growth and early embryo development.
- Further validation of the candidate markers proposed in this study using different populations and/ other stress relevant experimental model could lead to the development of markers which can be used as a foundation to enhance reproductive function in cows.
- In order to elucidate the interaction of miRNAs with the translation of their target genes, EV-coupled proteomic analysis of those different metabolic status cows in their follicular fluid and blood serum could be beneficial.
- Further in vitro study on the role of extracellular vesicles recovered from different metabolic status cows during oocyte maturation and embryo development could be beneficial to develop metabolic stress biomarkers miRNAs

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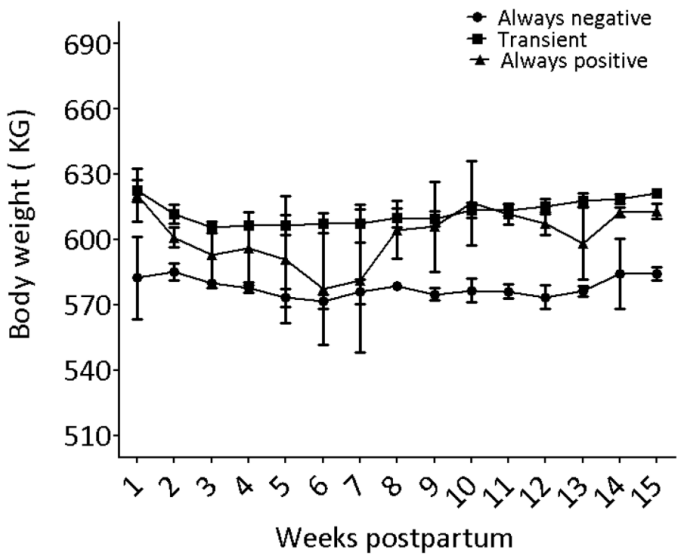
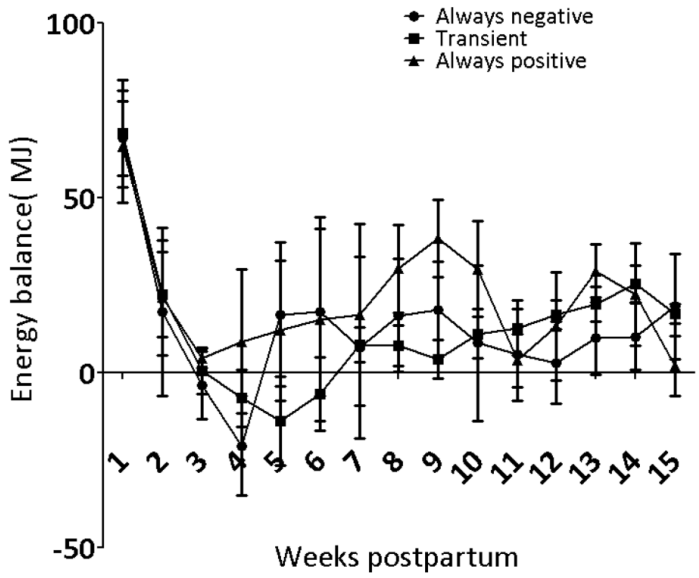
Chapter 5: List of appendixes

5.1 Appendix I: Supplementary data of chapter 2

5.1.1 List of supplementary figures

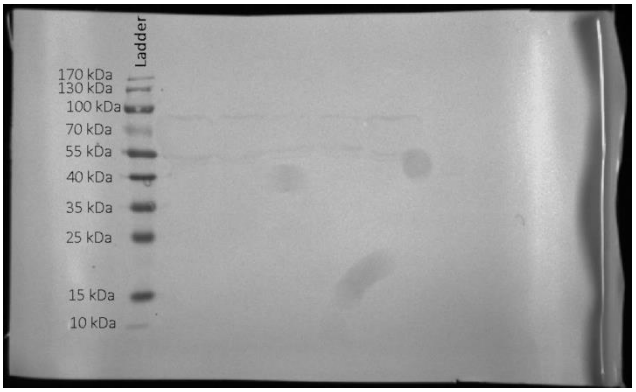


Supplementary fig. 5. 1.S1: Body weight curve of postpartum cows of different metabolic status.



Supplementary fig. 5. 2.S2: Over energy balance curve of postpartum cows based on their dry matter intake.

A)



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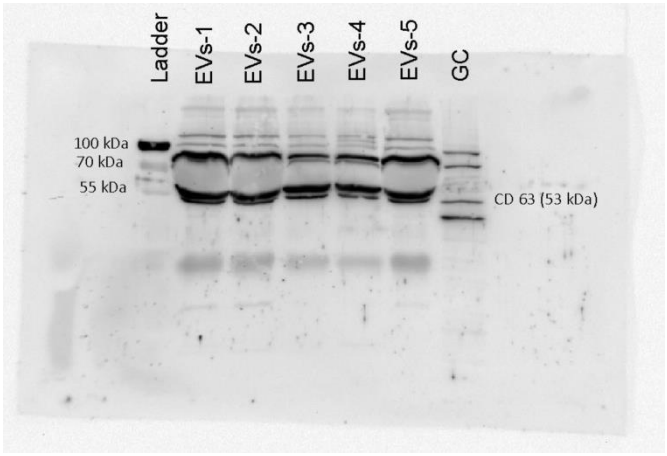

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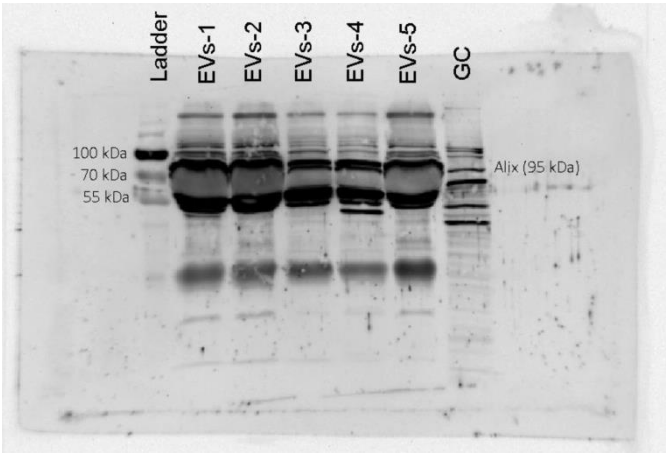
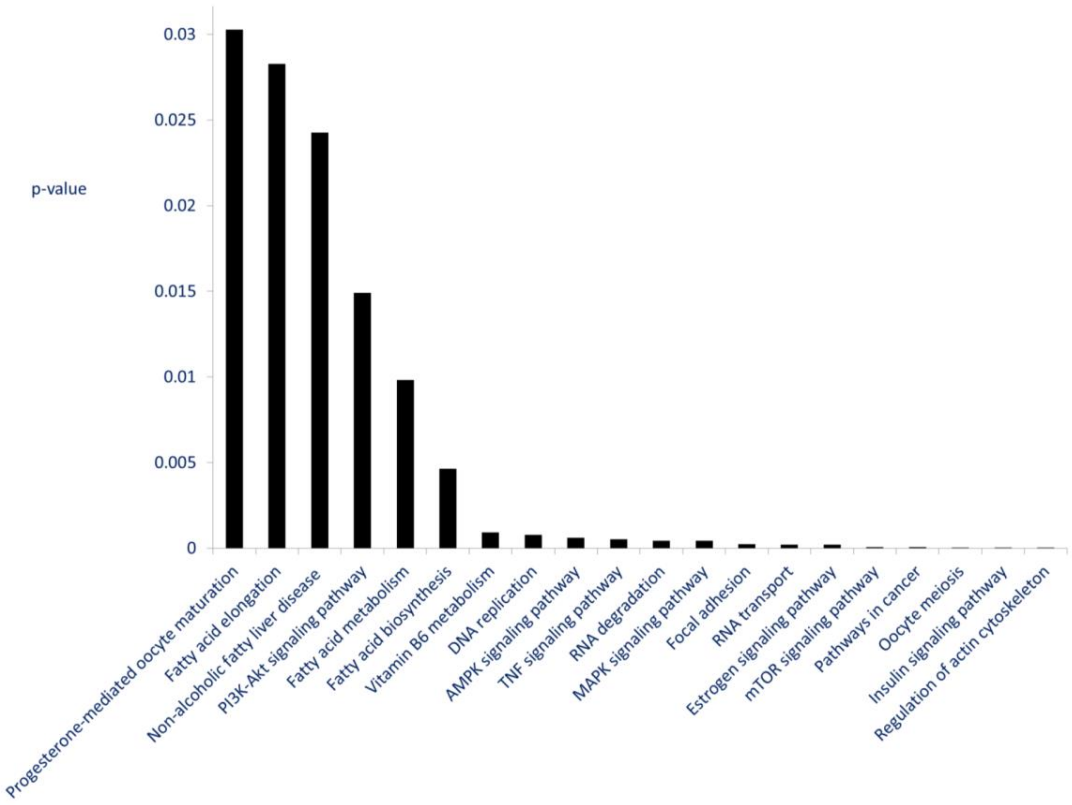
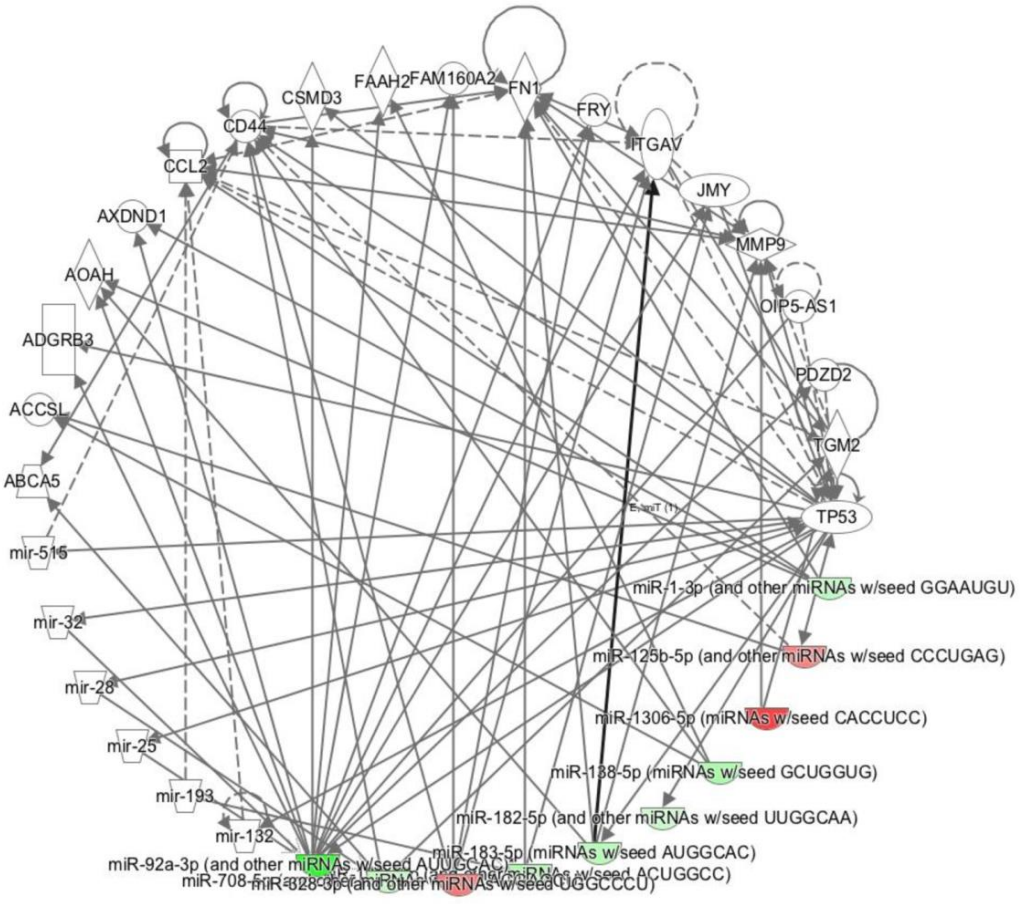


Supplementary fig. 5. 3.S3: Detection of protein markers using western blot in EVs recovered from different metabolic status cows: A) Ladder used for western blotting of protein detection for CD63 and Alix. B) Western blot showing CD63 protein detection in EVs recovered from follicular fluid of different metabolic status cows. C) Western blot showing the detection of Alix protein in EVs recovered from follicular fluid of different

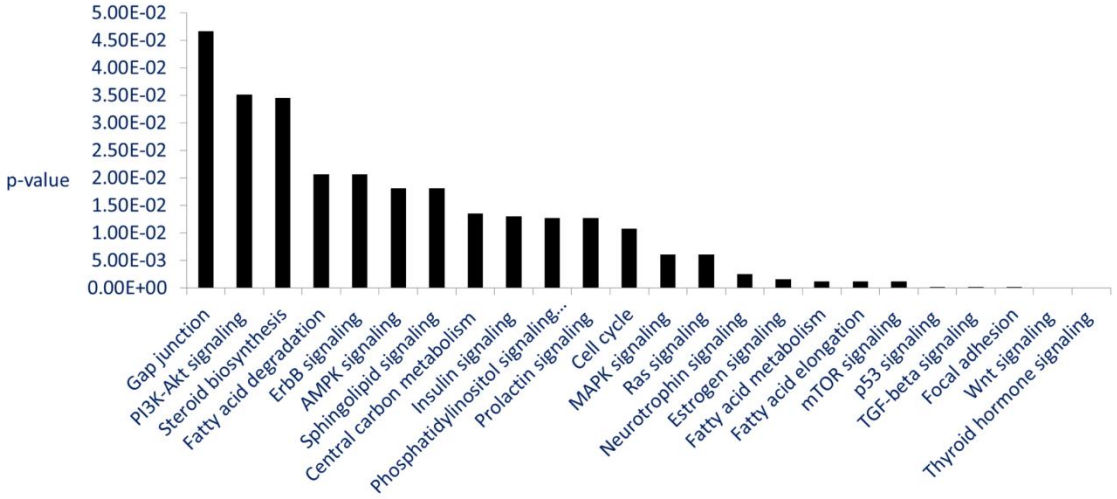
metabolic status cows. The numbers represent different sources EVs 1: EVs of TCs (weeks 5 - 6), 2: EVs of TCs (weeks 9 - 10), 3: EVs of ANCs, 4: EVs of APCs, 5: EVs of heifers and GC: Granulosa cells used as a control.



Supplementary fig. 5.4.S4: Target prediction analysis of downregulated miRNA in always negative cows vs heifers.



Supplementary fig. 5.5.S5: miRNA-gene network of differentially expressed miRNAs in always negative cows vs heifer.



Supplementary fig. 5.6.S6: Target prediction analysis of upregulated miRNAs in Always positive cows vs heifers upregulated

5.1.2 Appendix II: Supplementary tables

Supplementary table 5.1.S1: List of individual differentially expressed miRNA from comparison between Always negative and Always positive cows in follicular fluid with their corresponding log2 Fold change, p-value and false discovery rate (FDR) (top 100)

	Always negative	**Always positive**			
MiRNA name	**TMM**	**TMM**	**Log2 Fold change**	**P value**	**FDR**
bta-miR-451	15.8	426.5	-4.88936	0.000106161	0.028981917
bta-miR-132	79.21	2340.61	-4.87262	0.00052872	0.072170233
bta-miR-2285z	0	30.9	-6.07501	0.001121057	0.102016192
bta-miR-874	41.4	208.61	-2.37345	0.00330055	0.22526252
bta-miR-486	585.56	4739.52	-3.02148	0.004640482	0.253370306
bta-miR-34c	42.82	399.57	-3.17124	0.00650414	0.295938358
bta-miR-449a	12.64	153.7	-3.73106	0.007809762	0.30458073
bta-miR-1246	6691.51	30741.12	-2.20032	0.009344807	0.305558835
bta-miR-196a	80.19	15.79	2.30141	0.010746023	0.305558835
bta-miR-21-3p	5.01	41.16	-3.00641	0.011192631	0.305558835
bta-miR-150	148.17	536.37	-1.87498	0.01416989	0.351670917
bta-let-7e	4351.94	1868.31	1.21978	0.055293557	0.557240555
bta-miR-107	14473.7	41335.32	-1.51419	0.053761784	0.557240555
bta-miR-10a	1172.2	3351.18	-1.51602	0.08723129	0.557240555
bta-miR-1306	335.15	137.1	1.28987	0.074797825	0.557240555
bta-miR-135a	1.85	15.52	-2.53763	0.087820119	0.557240555

bta-miR-141	438.73	1803.67	-2.04251	0.051194078	0.557240555
bta-miR-142-5p	474.79	1269.77	-1.42549	0.067693443	0.557240555
bta-miR-147	8.17	30.5	-2.00092	0.080659562	0.557240555
bta-miR-196b	67.22	20.24	1.66454	0.062987148	0.557240555
bta-miR-197	75.29	22.4	1.73388	0.064404693	0.557240555
bta-miR-200a	2686.49	7254.65	-1.43349	0.093216868	0.557240555
bta-miR-200b	1139.08	3034.82	-1.4143	0.070892953	0.557240555
bta-miR-200c	769.74	2209.54	-1.52175	0.062637599	0.557240555
bta-miR-223	102.85	302.12	-1.57645	0.033368562	0.557240555
bta-miR-2284t-3p	44.13	11.2	2.00552	0.064419775	0.557240555
bta-miR-2285t	23.75	107.55	-2.16918	0.048886897	0.557240555
bta-miR-23a	16691.04	43358.09	-1.37745	0.066165697	0.557240555
bta-miR-23b-3p	13547.51	34821.14	-1.36221	0.072207546	0.557240555
bta-miR-27a-3p	388.18	1015.41	-1.39327	0.06239411	0.557240555
bta-miR-31	3234.55	1504.1	1.10496	0.082975877	0.557240555
bta-miR-328	386.9	158.01	1.29464	0.055041379	0.557240555
bta-miR-331-5p	6.86	29.55	-2.01057	0.092691405	0.557240555
bta-miR-362-3p	5.01	29.28	-2.51943	0.051141736	0.557240555
bta-miR-374a	105.79	34.27	1.60126	0.035204907	0.557240555
bta-miR-374b	55.46	15.11	1.84902	0.049090256	0.557240555
bta-miR-450b	1374.9	649.7	1.0822	0.093894013	0.557240555
bta-miR-502a	47.17	131.83	-1.54643	0.083693908	0.557240555
bta-miR-542-5p	326.53	118.2	1.46598	0.043483663	0.557240555
bta-miR-574	2397.54	1113.06	1.1072	0.080486408	0.557240555
bta-miR-652	94.46	281.2	-1.57955	0.02532204	0.557240555
bta-miR-6525	21.14	2.97	2.68207	0.031626429	0.557240555
bta-miR-671	123.65	340.44	-1.48383	0.045199924	0.557240555
bta-miR-7857	49.69	129.53	-1.31861	0.085155745	0.557240555
bta-miR-873	3364.75	8635.25	-1.36055	0.04393876	0.557240555
bta-miR-9-5p	61.66	13.9	2.05211	0.038988364	0.557240555
bta-miR-103	17302.2	40609.64	-1.23107	0.096368356	0.559756623
bta-miR-21-5p	51381.88	116142.62	-1.17658	0.098856505	0.562246372
bta-miR-199b	60.14	21.59	1.42697	0.101238912	0.564045369
bta-miR-190a	51.42	18.35	1.41386	0.103461029	0.564897218
bta-miR-22-3p	3851.27	8443.3	-1.13332	0.106640377	0.570839663
bta-miR-320a	64298.47	134557.61	-1.06541	0.112916911	0.592813782
bta-miR-424-5p	1702.74	852.24	0.998871	0.115565724	0.5952725
bta-miR-429	440.59	1009.9	-1.19898	0.122047517	0.617018005
bta-miR-335	661.03	1669.69	-1.33411	0.126018025	0.625507654
bta-miR-221	2018.46	4327.19	-1.10158	0.132314603	0.633717309
bta-miR-2299-3p	62.32	26.58	1.23967	0.130324222	0.633717309
bta-miR-6120-3p	28.76	69.22	-1.26606	0.138779582	0.653221138
bta-miR-130a	10000.29	21335.8	-1.09354	0.14962811	0.661467298
bta-miR-146a	70.92	175.96	-1.33664	0.155069257	0.661467298
bta-miR-27b	19559.09	10225.59	0.93576	0.149527527	0.661467298
bta-miR-320b	5.01	20.11	-1.98572	0.147180112	0.661467298

bta-miR-452	832	416.67	1.00102	0.150516375	0.661467298
bta-miR-876	35.08	12.28	1.43273	0.152840478	0.661467298
bta-miR-2284h-5p	6.32	21.32	-2.0006	0.159893441	0.670678618
bta-miR-497	1425.58	755.61	0.917045	0.164017788	0.670678618
bta-miR-6123	207.34	105.52	0.982691	0.164598782	0.670678618
bta-miR-2313-3p	7.41	25.23	-1.47272	0.168108272	0.67490527
bta-miR-18a	11.33	33.19	-1.67512	0.172967847	0.684351049
bta-miR-202	7062.25	3815.67	0.888093	0.178057871	0.694425698
bta-let-7f	24070.35	13526.78	0.831383	0.181217439	0.696259797
bta-miR-98	383.18	207.12	0.884878	0.183628958	0.696259797
bta-miR-182	203.31	399.02	-0.968004	0.213699083	0.757660384
bta-miR-2284z	316.29	174.74	0.85821	0.204008516	0.757660384
bta-miR-30c	128.02	68.41	0.901434	0.208038166	0.757660384
bta-miR-423-3p	4888.25	2748.28	0.83131	0.212970045	0.757660384
bta-miR-744	516.47	263.25	0.978936	0.209056483	0.757660384
bta-miR-1343-3p	511.42	284.99	0.839944	0.226006774	0.771237241
bta-miR-1388-5p	25.82	57.48	-1.2577	0.229629412	0.771237241
bta-miR-155	271.29	469.7	-0.793111	0.222712717	0.771237241
bta-miR-195	3537.42	2078.75	0.767259	0.231150451	0.771237241
bta-miR-3431	858.25	486.16	0.822925	0.231653677	0.771237241
bta-let-7i	27931.68	16031.38	0.801081	0.237412189	0.780885873
bta-miR-100	273.47	471.85	-0.784393	0.247289092	0.794234377
bta-miR-2483-3p	787.43	439.73	0.843535	0.244618624	0.794234377
bta-miR-494	51.21	104.04	-1.0187	0.250699772	0.795826022
bta-let-7c	7452.21	4565.54	0.706945	0.261851857	0.8046249
bta-miR-129-5p	69.73	126.16	-0.825658	0.277050332	0.8046249
bta-miR-190b	756.57	452.15	0.743181	0.25744143	0.8046249
bta-miR-2284x	7431.55	11964.22	-0.687131	0.272479782	0.8046249
bta-miR-2285aa	26.15	12.28	1.10229	0.27171441	0.8046249
bta-miR-2285o	15.03	4.45	1.55703	0.27645236	0.8046249
bta-miR-375	3407.58	7041.39	-1.04683	0.275062018	0.8046249
bta-miR-378c	164.19	282.69	-0.781283	0.265006019	0.8046249
bta-miR-1839	7543.7	12048.83	-0.675762	0.285855513	0.812901615
bta-miR-6517	101.11	171.91	-0.758043	0.283249664	0.812901615
bta-miR-143	23221.77	36148.8	-0.638518	0.311278355	0.816767464
bta-miR-181a	908.43	1456.77	-0.68274	0.293932385	0.816767464
bta-miR-183	147.52	243.7	-0.727396	0.315082686	0.816767464
bta-miR-191	6584.8	10782.86	-0.711243	0.290356557	0.816767464

Supplementary table 5.2.S2: List of individual differentially expressed miRNA from comparison between Always negative cows and heifers in follicular fluid with their corresponding log2 Fold change, p-value and false discovery rate (FDR) (top 100)

MiRNA name	Always negative	Heifers	Log2 Fold	P value	FDR

	TMM	TMM	change		
bta-miR-223	125.45	875.5	-2.84357	1.5046e-5	0.004453529
bta-miR-132	93.73	2719.73	-4.84899	0.000105994	0.01123324
bta-miR-150	181.84	914.97	-2.37011	0.000151801	0.01123324
bta-miR-451	20.05	228.61	-3.76768	0.000119314	0.01123324
bta-miR-155	325.07	1375.11	-2.08713	0.000551881	0.023749681
bta-miR-184	174.65	2454.35	-3.84116	0.000514664	0.023749681
bta-miR-424-5p	2015.21	654.03	1.6207	0.000561648	0.023749681
bta-miR-200c	924.02	2805.82	-1.60655	0.001143047	0.042292727
bta-miR-142-5p	583.69	2285.45	-1.98137	0.001564631	0.044016204
bta-miR-146a	86.91	376.36	-2.18046	0.001366765	0.044016204
bta-miR-200b	1370.16	4067.04	-1.57274	0.001635737	0.044016204
bta-miR-122	1924.24	12098.39	-2.65533	0.003596237	0.079172158
bta-miR-18a	14.1	76.19	-2.65757	0.003479496	0.079172158
bta-miR-20b	0	21.23	-5.5739	0.003744629	0.079172158
bta-miR-183	176.76	619.32	-1.81961	0.004943991	0.097561429
bta-miR-1306	393.91	139.79	1.51161	0.005566749	0.098176065
bta-miR-363	0	18.68	-5.33923	0.005892563	0.098176065
bta-miR-542-5p	388.66	151.21	1.35131	0.005970166	0.098176065
bta-miR-147	10.09	56.61	-2.6549	0.006944787	0.108192476
bta-miR-205	1000.1	3541.22	-1.83153	0.007895656	0.116855705
bta-miR-182	240.47	598.4	-1.31088	0.009461357	0.133360086
bta-miR-138	6.2	38.39	-2.16905	0.012053609	0.162175836
bta-miR-206	30.76	109.02	-1.70096	0.015119224	0.172126545
bta-miR-450b	1624.61	776.18	1.06552	0.0141946	0.172126545
bta-miR-6518	71.36	21.97	1.69812	0.013996998	0.172126545
bta-miR-92a	60915.34	27486.24	1.1481	0.014542944	0.172126545
bta-miR-191	7774.03	17291.16	-1.15298	0.018071338	0.198115408
bta-miR-26a	14872.37	7174.03	1.05137	0.02043771	0.199099788
bta-miR-328	455.07	215.61	1.08479	0.020851667	0.199099788
bta-miR-423-5p	62976.07	30282.96	1.05625	0.019371241	0.199099788
bta-miR-432	279.09	738.26	-1.42598	0.020244719	0.199099788
bta-miR-708	30.51	93.28	-1.62365	0.02258893	0.2089476
bta-miR-125a	11060.62	5530.14	1.00014	0.025168439	0.214657563
bta-miR-193b	4.13	26.48	-2.15027	0.024162667	0.214657563
bta-miR-92b	6096.23	2558.5	1.25194	0.025381806	0.214657563
bta-miR-21-3p	6.08	37.25	-2.58832	0.029057674	0.232461396
bta-miR-6520	117.92	46.65	1.30232	0.02875309	0.232461396
bta-miR-1839	9106.5	3971.19	1.19648	0.032269085	0.251359186
bta-miR-2313-3p	8.27	34.68	-1.65941	0.033687458	0.252369812
bta-miR-486	733.99	2495.07	-1.77336	0.034104029	0.252369812
bta-miR-21-5p	61617.52	122250.69	-0.988499	0.039944131	0.283885797
bta-miR-365-5p	2.07	18.45	-2.47734	0.041170728	0.283885797
bta-miR-9-5p	76.57	24.7	1.51719	0.041240166	0.283885797
bta-miR-197	87.9	37.74	1.24539	0.044234888	0.297580153

bta-miR-16b	36136.8	67398.73	-0.899206	0.051152844	0.317398571
bta-miR-2299-3p	73.43	31.4	1.28313	0.054793779	0.317398571
bta-miR-2387	45.23	116.86	-1.22676	0.052849573	0.317398571
bta-miR-27b	22940.22	12320.76	0.896884	0.055759208	0.317398571
bta-miR-31	3815.42	2128.7	0.842583	0.048808833	0.317398571
bta-miR-320a	78434.76	36346.68	1.10957	0.055602243	0.317398571
bta-miR-34c	49.24	219.18	-2.10494	0.054850472	0.317398571
bta-miR-503-5p	132.77	305.53	-1.18857	0.055553283	0.317398571
bta-miR-130a	12279.15	4942.7	1.31205	0.060415284	0.325144073
bta-miR-455-5p	34.77	93.8	-1.34255	0.059544927	0.325144073
bta-miR-502a	58.46	18.61	1.49159	0.058224704	0.325144073
bta-miR-125b	6735.35	3863.45	0.801667	0.061586273	0.325527444
bta-miR-186	615.04	1113.77	-0.856104	0.064121638	0.327241463
bta-miR-760-3p	1557.21	821.47	0.919177	0.063220023	0.327241463
bta-miR-6120-3p	34.52	81.16	-1.2873	0.067047991	0.33637636
bta-miR-429	531.47	1043.38	-0.982885	0.068623297	0.336781362
bta-miR-592	65.29	28.23	1.21695	0.069404267	0.336781362
bta-miR-154c	26.38	63.37	-1.32679	0.076318479	0.34754261
bta-miR-192	5767.76	12674.85	-1.13681	0.075709645	0.34754261
bta-miR-23b-5p	40.73	16.04	1.30903	0.073126267	0.34754261
bta-miR-6528	4.13	21.09	-1.89985	0.076124107	0.34754261
bta-miR-222	111.22	231.14	-1.10839	0.079833318	0.358040336
bta-miR-181a	1093.83	1956.7	-0.84256	0.081717849	0.36102214
bta-miR-1343-3p	613.42	348.81	0.805418	0.104725935	0.374287065
bta-miR-1343-5p	14.47	44.72	-1.30051	0.103249435	0.374287065
bta-miR-1388-5p	32.21	72.99	-1.35409	0.111274533	0.374287065
bta-miR-141	537.63	1045.97	-0.975109	0.09886497	0.374287065
bta-miR-190a	62.72	26.16	1.11554	0.100464294	0.374287065
bta-miR-200a	3243.05	5711.5	-0.818202	0.11003137	0.374287065
bta-miR-221	2468.9	4600.64	-0.901055	0.100959106	0.374287065
bta-miR-2284t-3p	51.19	24.53	1.17493	0.109957147	0.374287065
bta-miR-2284x	8932.84	5302.71	0.751811	0.106539518	0.374287065
bta-miR-2284z	374.81	216.08	0.792111	0.089871342	0.374287065
bta-miR-2285o	18.23	4.5	1.84247	0.100109861	0.374287065
bta-miR-2320-5p	2.07	14.19	-2.2056	0.099540618	0.374287065
bta-miR-2483-3p	921.46	538.01	0.782477	0.109983024	0.374287065
bta-miR-323	2.07	13.48	-2.18331	0.099735725	0.374287065
bta-miR-361	26102.31	13453.96	0.955844	0.088889601	0.374287065
bta-miR-383	2.07	13.48	-2.18331	0.099735725	0.374287065
bta-miR-424-3p	93.61	46.71	0.964552	0.086656195	0.374287065
bta-miR-497	1677.28	944.12	0.830493	0.092205695	0.374287065
bta-miR-6123	243.04	140.32	0.811243	0.108846573	0.374287065
bta-miR-628	4.13	17.29	-1.62906	0.108831771	0.374287065
bta-miR-7860	32.58	10.86	1.64536	0.087845443	0.374287065
bta-miR-202	8462.03	5082.13	0.735142	0.117816347	0.391838636

bta-let-7g	4029.18	6660.27	-0.724669	0.124601782	0.392363058
bta-miR-144	0	7.85	-4.06512	0.121873681	0.392363058
bta-miR-23b-3p	16745.1	8419.99	0.9914	0.120748221	0.392363058
bta-miR-296-3p	4260.06	2638.53	0.690599	0.123854587	0.392363058
bta-miR-99b	2080.66	1227.81	0.758996	0.122540545	0.392363058
bta-let-7b	50829.42	82679.92	-0.70185	0.14714122	0.423387298
bta-miR-10a	1413.9	2377.97	-0.753108	0.150154953	0.423387298
bta-miR-127	28.32	59.51	-1.18335	0.146752015	0.423387298
bta-miR-194	1047.56	2008.17	-0.943661	0.142500942	0.423387298
bta-miR-199c	156.58	279.26	-0.82932	0.145774543	0.423387298
bta-miR-2284ab	1729.05	1070.33	0.690725	0.137557844	0.423387298

Supplementary table 5.2.S3: List of individual differentially expressed miRNA from comparison between Always positive cows and heifers in follicular fluid with their corresponding log2 Fold change, p-value and false discovery rate (FDR) top 100

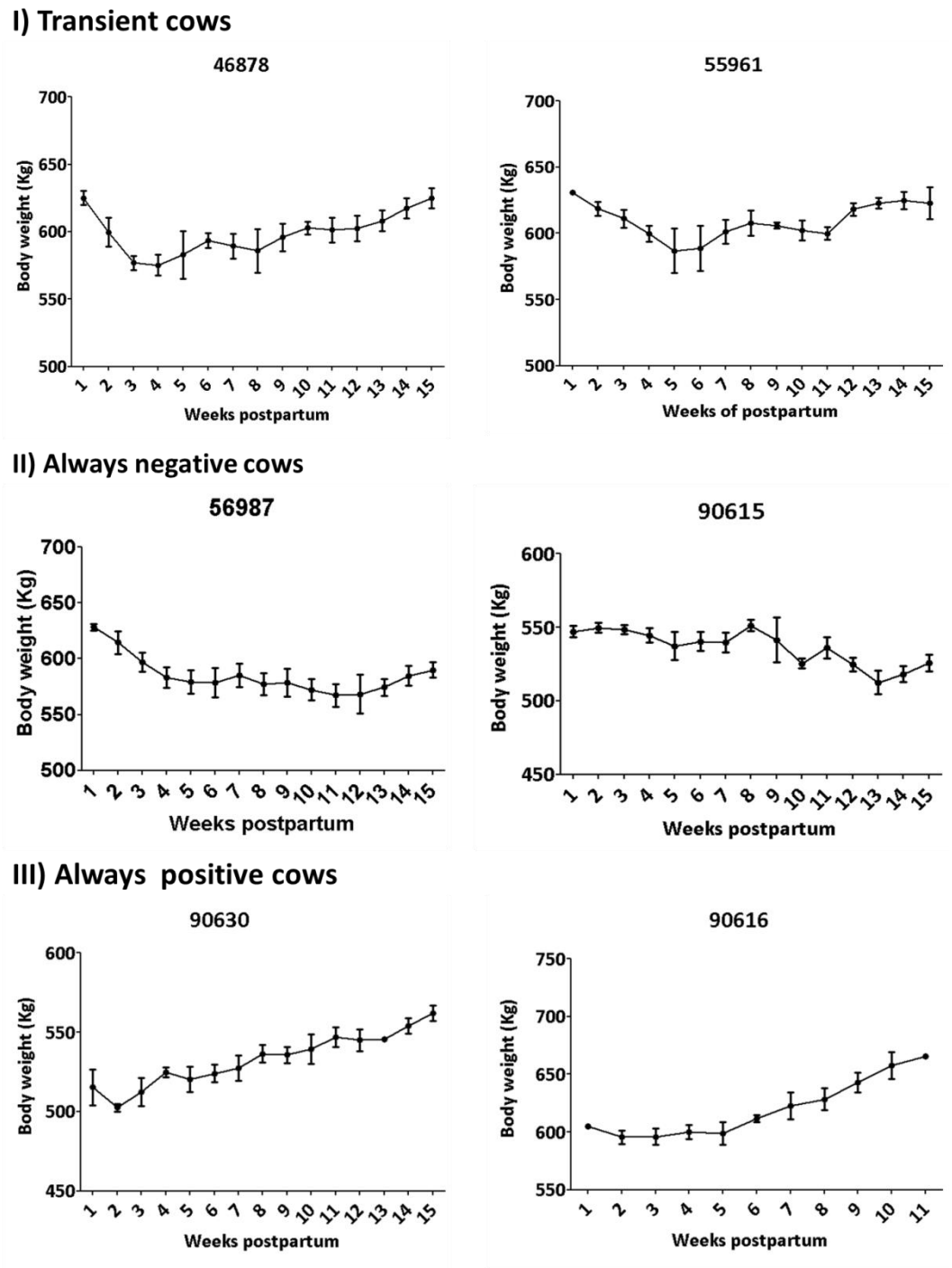
MiRNA name	**Always positive TMM**	**Heifers TMM**	**Log2 Fold change**	**P value**	**FDR**
bta-miR-1246	33974.57	4846.76	2.80911	2.29E-06	0.000344242
bta-miR-874	230.75	30.7	2.94159	1.79E-02	0.000344242
bta-miR-184	140.03	2137.16	-3.93165	7.22E-06	0.000721578
bta-miR-502a	145.84	16.32	3.09418	1.48E-05	0.001113181
bta-miR-23b-3p	38460.93	7357.16	2.38612	2.70E-05	0.00161719
bta-miR-107	45710.3	9316.24	2.29459	8.29E-05	0.003800784
bta-miR-2285z	34.14	2.38	3.45259	0.000114024	0.003800784
bta-miR-23a	47898.5	11874.35	2.01212	0.000101565	0.003800784
bta-miR-320a	148650.4	31784.41	2.2255	0.000113902	0.003800784
bta-miR-196a	17.38	152.79	-3.12224	0.000286395	0.008591851
bta-miR-103	44908.49	10770.95	2.05977	0.000324221	0.008669
bta-miR-22-3p	9333.16	2434.79	1.93847	0.00034676	0.008669
bta-miR-130a	23627.76	4333.53	2.44649	0.000672041	0.015508637
bta-miR-1839	13301.78	3474.36	1.9364	0.000932599	0.019984269
bta-miR-423-5p	82036.05	26419.48	1.63464	0.001122037	0.022440743
bta-miR-205	895.08	3078.37	-1.78152	0.00220854	0.041410123
bta-miR-26a	17422.87	6256.15	1.47755	0.002418248	0.042674969
bta-miR-196b	22.33	130.08	-2.53311	0.003193341	0.043545558
bta-miR-2284x	13219.16	4627.82	1.51409	0.002751014	0.043545558
bta-miR-331-5p	32.61	5.64	2.44125	0.003113762	0.043545558
bta-miR-6520	145.68	40.8	1.81295	0.002945993	0.043545558
bta-miR-671	376.02	129.93	1.52548	0.002945978	0.043545558
bta-miR-652	310.1	105.7	1.54738	0.003526411	0.045996669
bta-miR-135a	17.13	0.86	3.72497	0.003899178	0.048739724
bta-miR-362-3p	32.36	5.61	2.28075	0.004499912	0.053998943
bta-miR-365-5p	0	16.04	-5.33756	0.005681515	0.065555944

bta-miR-873	9540.4	2009.85	2.24622	0.00770397	0.085599665
bta-miR-2366	95.69	28.92	1.68232	0.008390744	0.08841039
bta-miR-320b	22.21	3.76	2.7526	0.008546338	0.08841039
bta-miR-6517	189.85	70.22	1.45612	0.011299963	0.10935448
bta-miR-6518	66.63	19.22	1.77872	0.011051925	0.10935448
bta-miR-1291	17.13	2.31	2.91636	0.012358519	0.112350175
bta-miR-194	575.71	1742.56	-1.59643	0.012006649	0.112350175
bta-miR-204	44.67	126.49	-1.49621	0.016318077	0.143983034
bta-miR-449a	170.79	21.4	2.94514	0.023970269	0.205459445
bta-miR-92b	5913.8	2234.63	1.40416	0.025143112	0.209525935
bta-miR-296-3p	4994.23	2302.28	1.11695	0.026079316	0.211453913
bta-miR-335	1834.38	614.55	1.57936	0.029174021	0.23032122
bta-miR-192	4055.99	10999.7	-1.43909	0.041776072	0.321354399
bta-miR-223	333.91	761.43	-1.19035	0.043462999	0.325972493
bta-miR-206	36.04	95.26	-1.39478	0.044860697	0.327085043
bta-miR-361	26878.76	11769.69	1.19129	0.045791906	0.327085043
bta-miR-2284v	15.23	3.76	2.22466	0.047269793	0.329789251
bta-miR-155	518.36	1202.46	-1.2149	0.049169612	0.335247355
bta-miR-122	3222.41	10497.67	-1.70351	0.051517263	0.343448417
bta-miR-486	5210.81	2167.99	1.26567	0.053627919	0.3497473
bta-miR-2285t	119.49	40.91	1.53073	0.060057498	0.375359364
bta-miR-2320-5p	1.65	12.35	-2.60456	0.059593661	0.375359364
bta-miR-151-5p	213.95	106	1.00464	0.064984134	0.389904803
bta-miR-30b-5p	20.56	52.36	-1.3297	0.063752887	0.389904803
bta-miR-342	3317.99	6404.85	-0.948746	0.067173536	0.395138449
bta-miR-145	2174.69	1051.19	1.04832	0.073099548	0.413771027
bta-miR-7857	142.87	71.34	0.993108	0.072478715	0.413771027
bta-miR-185	1953.21	1049.11	0.897397	0.075534706	0.419637255
bta-miR-451	468.66	198.47	1.24642	0.088578146	0.483153523
bta-miR-6525	3.3	14.06	-1.94065	0.090961477	0.487293629
bta-let-7e	2065.43	3776.73	-0.870299	0.093469764	0.491253636
bta-miR-2284t-5p	126.36	61.04	1.0139	0.094975703	0.491253636
bta-miR-27a-3p	1124.56	604.55	0.895586	0.098391122	0.500293841
bta-miR-2285k	58.62	28.72	1.02428	0.111402558	0.54788143
bta-miR-365-3p	23.98	52.45	-1.13147	0.111315652	0.54788143
bta-miR-379	113.19	216.87	-0.934214	0.114171001	0.552440328
bta-miR-22-5p	150.66	79.94	0.920381	0.116757888	0.555989943
bta-miR-190b	498.51	884.61	-0.827344	0.123500028	0.570860606
bta-miR-193a-5p	2188.38	1242.06	0.816541	0.126189222	0.570860606
bta-miR-450a	229.54	92.06	1.30456	0.127122311	0.570860606
bta-miR-760-3p	1272.92	717.69	0.824067	0.127492202	0.570860606
bta-miR-218	56.47	27.18	0.983991	0.131223745	0.578928288
bta-let-7g	3357.23	5789.96	-0.786198	0.147896323	0.621532389
bta-miR-1	299.2	533.46	-0.835477	0.144226411	0.621532389
bta-miR-183	269.87	541.96	-1.00658	0.149113225	0.621532389
bta-miR-3956	1.78	9.87	-2.24693	0.149167773	0.621532389
bta-miR-141	2004.04	911.23	1.13615	0.153740969	0.62160122
bta-miR-20b	6.85	18.57	-1.47252	0.157581173	0.62160122

bta-miR-411c-5p	1.78	9.68	-2.23386	0.159558022	0.62160122
bta-miR-424-5p	941.1	571.01	0.718286	0.161616317	0.62160122
bta-miR-499	44.67	22.38	0.974286	0.151885361	0.62160122
bta-miR-628	4.95	15.11	-1.50679	0.156548161	0.62160122
bta-miR-2284aa	139.85	81.22	0.762541	0.186080047	0.697800175
bta-miR-425-5p	1388.41	2205.39	-0.667447	0.185939076	0.697800175
bta-miR-215	1452.01	2701.94	-0.895041	0.200514059	0.699467649
bta-miR-222	120.82	200.97	-0.730813	0.19983332	0.699467649
bta-miR-301a	69.77	127.44	-0.870899	0.199791263	0.699467649
bta-miR-369-5p	17.13	35.59	-1.04399	0.1915516	0.699467649
bta-miR-378c	312.89	192.06	0.70921	0.193589061	0.699467649
bta-miR-7860	22.58	9.5	1.29747	0.196970748	0.699467649
bta-miR-140	1296.49	2085.83	-0.684957	0.205965473	0.710225769
bta-miR-92a	38184.96	23997.9	0.670083	0.208992574	0.712474683
bta-miR-10a	3720.54	2066.36	0.848405	0.231646493	0.71832692
bta-miR-125a	7573.94	4824.1	0.650791	0.224171078	0.71832692
bta-miR-126-3p	1360.91	897.45	0.602054	0.232047188	0.71832692
bta-miR-130b	893.62	563.59	0.66837	0.228323248	0.71832692
bta-miR-138	15.23	33.32	-1.0498	0.227952174	0.71832692
bta-miR-16b	37522.97	58615.38	-0.643505	0.241143497	0.71832692
bta-miR-18a	36.54	66.26	-0.854516	0.218182194	0.71832692
bta-miR-214	978.59	1511.2	-0.626577	0.239593627	0.71832692
bta-miR-224	1256.64	1921.05	-0.612086	0.245882081	0.71832692
bta-miR-374a	37.81	64.78	-0.774705	0.246625576	0.71832692
bta-miR-374b	16.76	34.45	-1.0029	0.232029557	0.71832692
bta-miR-375	7822.53	4142.57	0.91718	0.242022396	0.71832692

5.2 Appendix III: Supplementary data of chapter 3

5.2.1 List of supplementary figures



Supplementary fig. 5. 3.S1: Representative individual body weight of lactating cows. The number on the top shows the ID number of the representative cows.

Supplementary table 5.3.S1: List of individual differentially expressed miRNA from comparison between Always negative cows at week 5-6 and heifers in blood serum with their corresponding log2 Fold change, p-value and false discovery rate (FDR) (top 100)

miRNA name	Always negative : week 5-6 TMM	Heifers TMM	Log2 Fold change	P value	FDR
bta-miR-122	2462.05	14519.69	-2.55716	1.87E-07	5.12E-05
bta-miR-375	233.83	1195.58	-2.3381	3.95E-06	0.000541349
bta-miR-192	3206.73	10307.4	-1.6851	3.34E-05	0.00304805
bta-miR-194	541.96	2008.51	-1.88682	5.88E-05	0.004026711
bta-miR-107	687.18	2136.34	-1.63264	9.86E-05	0.004239158
bta-miR-214	81	555.24	-2.75716	0.000100222	0.004239158
bta-miR-215	1144.56	3882.27	-1.76362	0.0001083	0.004239158
bta-miR-9-5p	1066.6	31.93	4.96909	0.000172732	0.005916054
bta-miR-145	107.79	436.23	-2.02577	0.000518268	0.015778387
bta-miR-140	1029.07	3152.57	-1.60759	0.000843383	0.023108693
bta-miR-143	4110.74	12497.15	-1.60534	0.001109134	0.02595659
bta-miR-184	1788.92	77.8	4.53191	0.001136785	0.02595659
bta-miR-199c	52.38	255.47	-2.29076	0.001731771	0.036500409
bta-miR-455-5p	0	87.79	-5.60747	0.003567923	0.069829357
bta-miR-30d	14515.27	6516.81	1.15255	0.004456835	0.081411522
bta-miR-141	51.46	207.9	-2.00212	0.005285515	0.09051445
bta-miR-130a	86.78	287.08	-1.70881	0.008055997	0.129158774
bta-miR-362-5p	84.03	10.64	2.76433	0.008484883	0.129158774
bta-miR-223	48927.86	24716.81	0.984507	0.009896412	0.142716678
bta-miR-2285p	123.93	25.02	2.11883	0.011231559	0.146545099
bta-miR-320a	2835.94	5763.54	-1.02289	0.011113444	0.146545099
bta-miR-378	403.44	911.79	-1.17439	0.012186389	0.151775941
bta-miR-26b	49187.27	27214.97	0.853563	0.014479903	0.17249971
bta-miR-652	256.67	92.49	1.50607	0.016167493	0.184578879
bta-miR-10a	3170.42	1464.66	1.11496	0.020733262	0.21040421
bta-miR-204	295.93	115.66	1.32187	0.020580151	0.21040421
bta-miR-374a	623.88	293.23	1.09156	0.019936281	0.21040421
bta-miR-197	299.32	114.9	1.328	0.024727742	0.211731295
bta-miR-199a-3p	10814.12	27264.23	-1.33381	0.024445241	0.211731295
bta-miR-224	249.97	546.65	-1.12857	0.023550948	0.211731295
bta-miR-2448-3p	160.17	46.24	1.79892	0.022447721	0.211731295
bta-miR-98	2248.85	1198.3	0.897518	0.023868655	0.211731295
bta-miR-1246	744.87	3996.76	-2.42096	0.028528994	0.231397637
bta-miR-1468	3866.84	2166.91	0.839672	0.028713575	0.231397637
bta-miR-1306	694.51	336.33	1.00064	0.032166327	0.251816388
bta-miR-142-3p	3381.76	1890.34	0.830715	0.037909319	0.287007444

bta-miR-151-3p	7489.57	4366.36	0.775699	0.039803952	0.287007444
bta-miR-425-5p	9732.2	5385.27	0.850778	0.03881148	0.287007444
bta-miR-15a	610.76	1126.28	-0.87638	0.041054151	0.288431727
bta-miR-150	3178.04	6440.57	-1.01666	0.043151798	0.295045811
bta-miR-30e-5p	6859.38	4193.24	0.709115	0.044149191	0.295045811
bta-let-7f	86231.54	51446.21	0.744953	0.046048612	0.300412371
bta-miR-152	997.23	2036.85	-1.02838	0.049844194	0.317611839
bta-miR-200b	49.63	142.78	-1.47704	0.053695714	0.334377856
bta-miR-211	43.85	3.74	2.63174	0.055160331	0.335865126
bta-miR-193a-5p	102.01	254.02	-1.28798	0.057930122	0.345062031
bta-miR-34a	98.34	255.56	-1.40798	0.06061475	0.353371098
bta-miR-2285k	28.62	0	3.98244	0.062652538	0.353460489
bta-miR-7859	29.54	0	3.99188	0.063210087	0.353460489
bta-miR-126-3p	181054.9	111230.1	0.702996	0.067865856	0.357600857
bta-miR-185	1215.29	2479.27	-1.02475	0.06757324	0.357600857
bta-miR-505	87.97	21.85	1.73631	0.065793595	0.357600857
bta-miR-128	4137.54	2538.77	0.698425	0.076229127	0.386792237
bta-miR-200a	114.48	243.87	-1.09549	0.075021046	0.386792237
bta-miR-1388-3p	42.93	114.03	-1.33758	0.1014709	0.427738873
bta-miR-147	20.09	71.77	-1.65909	0.095360203	0.427738873
bta-miR-155	3621.73	2395.14	0.596914	0.09910369	0.427738873
bta-miR-2284w	74.3	178.89	-1.26584	0.089039042	0.427738873
bta-miR-2284x	1372.52	2183.66	-0.66875	0.093129371	0.427738873
bta-miR-2419-5p	650.58	336.11	0.953278	0.089803329	0.427738873
bta-miR-26a	48576.2	32993.88	0.558008	0.098239794	0.427738873
bta-miR-27a-3p	2032.36	1288.75	0.658168	0.088741106	0.427738873
bta-miR-421	419.86	236.91	0.815714	0.096914021	0.427738873
bta-miR-7	981.83	518.06	0.910754	0.101070112	0.427738873
bta-miR-744	201.26	93.31	1.09711	0.098301847	0.427738873
bta-miR-30c	1376.74	838.23	0.712489	0.105364593	0.430894007
bta-miR-628	35.32	7.16	2.32901	0.104361348	0.430894007
bta-miR-31	76.41	28.44	1.43775	0.117530037	0.473576916
bta-miR-423-3p	2764.02	1867.55	0.561983	0.126664443	0.501245263
bta-miR-486	15382.69	24900	-0.69431	0.128055359	0.501245263
bta-miR-29b	1494.98	982.94	0.594826	0.135024587	0.521080802
bta-let-7a-5p	95569.56	66989.39	0.512576	0.139461611	0.526799159
bta-miR-126-5p	47598.12	32595.69	0.546559	0.142237262	0.526799159
bta-miR-23a	10274.42	15373.89	-0.58118	0.142274225	0.526799159
bta-miR-30a-5p	3847.96	2557.73	0.589882	0.148605075	0.542903873
bta-miR-151-5p	617.46	394.32	0.642991	0.153796387	0.547275457
bta-miR-25	9603	15381.66	-0.67919	0.152054805	0.547275457
bta-miR-103	16336.92	11473.82	0.508912	0.162612689	0.547940835
bta-miR-1307	952.1	617.44	0.601645	0.163982294	0.547940835
bta-miR-30b-5p	437.48	234.48	0.857726	0.156268872	0.547940835
bta-miR-532	229.24	413.59	-0.81963	0.158849354	0.547940835

bta-miR-6529	1576.9	991.48	0.659056	0.162286329	0.547940835
bta-miR-2478	313.27	157.38	0.903064	0.169189295	0.558528516
bta-miR-148a	6379.67	10241.32	-0.68226	0.175203393	0.564733163
bta-miR-32	265.48	437.9	-0.70952	0.177252015	0.564733163
bta-miR-331-3p	59.99	21.54	1.34675	0.174905001	0.564733163
bta-miR-424-5p	0	25.27	-3.89833	0.179943003	0.566717045
bta-miR-455-3p	0	22.1	-3.86403	0.18232852	0.567704711
bta-let-7d	3735.48	2605.76	0.517405	0.187692429	0.571419172
bta-miR-2346	7.61	42.26	-2.17852	0.186510204	0.571419172
bta-miR-1271	87.7	36.48	1.02478	0.192297558	0.575343424
bta-miR-454	563.25	353.89	0.660615	0.193181004	0.575343424
bta-let-7i	25557.68	18845.18	0.439149	0.208032791	0.588795233
bta-miR-15b	955.96	1453.26	-0.59784	0.208442108	0.588795233
bta-miR-195	469.49	732.94	-0.62408	0.206014449	0.588795233
bta-miR-2336	29.54	6.91	2.03975	0.200798079	0.588795233
bta-miR-664b	33.48	3.74	2.35148	0.205041566	0.588795233
bta-miR-339a	429.87	264.78	0.709841	0.211451146	0.591200142
bta-miR-210	14.31	56.32	-1.71938	0.230200505	0.619100817
bta-miR-221	6368.32	4671.46	0.44425	0.233883992	0.619100817

Supplementartry table 5.3.S2: List of individual differentially expressed miRNA from comparison between Transient cow at week 5-6 and heifers cows in blood serum with their corresponding log2 Fold change, p-value and false discovery rate (FDR) (top 100)

MiRNA names	Transient cows: week 5-6 TMM	Heifers TMM	Log2 Fold change	P value	FDR
bta-miR-215	167.2	3761.91	-4.54603	1.0243e-24	2.70E-22
bta-miR-192	1084.91	9988	-3.20598	5.9497e-23	7.85E-21
bta-miR-122	747.12	14068.48	-4.22894	1.2578e-22	1.11E-20
bta-miR-143	1864.95	12110.07	-2.70324	9.1506e-19	6.04E-17
bta-miR-375	71.44	1158.44	-4.12767	9.5952e-17	5.07E-15
bta-miR-107	374.24	2070.05	-2.4585	1.4358e-14	6.32E-13
bta-miR-194	365.03	1946.18	-2.41155	2.4954e-11	9.41E-10
bta-miR-214	30.91	537.96	-3.94985	5.5087e-11	1.82E-09
bta-miR-378	171.46	883.47	-2.35854	9.2224e-10	2.71E-08
bta-miR-206	93.02	3794.38	-5.35412	2.6148e-8	6.90E-07
bta-miR-145	73.37	422.72	-2.44903	5.8863e-7	1.30E-05
bta-miR-199c	27.46	247.54	-3.21138	5.5542e-7	1.30E-05
bta-miR-199a-3p	6982.97	26415.07	-1.91836	1.0297e-6	2.09E-05
bta-miR-320a	2172.51	5584.6	-1.35952	1.3856e-6	2.61E-05
bta-miR-130a	47.43	278.16	-2.54747	3.5586e-6	6.21E-05
bta-miR-133a	43.98	1081.26	-4.68326	3.7609e-6	6.21E-05

bta-miR-193a-5p	37.19	246.12	-2.63472	7.4433e-6	0.00011559
bta-miR-140	968.47	3054.47	-1.64392	1.4339e-5	0.00021031
bta-miR-152	761.06	1973.52	-1.37539	0.00014088	0.00185968
bta-miR-382	35.27	206.53	-2.27951	0.00013797	0.00185968
bta-miR-1468	4403.55	2099.53	1.07526	0.0002476	0.0031127
bta-miR-1	136.29	2292.42	-4.12449	0.00029345	0.00352141
bta-miR-652	308.09	89.61	1.81262	0.00063603	0.00730052
bta-miR-338	289.42	90.72	1.63218	0.00071757	0.00789329
bta-miR-1246	526.27	3871.8	-2.88255	0.00080339	0.00848378
bta-miR-30e-5p	7286.54	4063.14	0.840894	0.00088662	0.00900259
bta-miR-26b	47346.98	26371	0.844129	0.00110853	0.01083894
bta-miR-30d	13603.08	6315.16	1.10463	0.00119857	0.0113008
bta-miR-378c	6.59	79.48	-3.63642	0.00202862	0.01846745
bta-miR-223	45477.56	23951.47	0.924195	0.00214137	0.01884406
bta-miR-2284w	50.88	173.33	-1.65296	0.0030882	0.02629947
bta-miR-126-3p	200569.59	107769.89	0.896311	0.00583988	0.04785134
bta-miR-26a	49852.98	31969.83	0.641053	0.00598142	0.04785134
bta-miR-142-3p	3297.37	1831.82	0.83399	0.00718679	0.0542089
bta-miR-425-5p	10053.52	5218.6	0.943573	0.00718414	0.0542089
bta-miR-30a-5p	4568.66	2478.33	0.884506	0.00782911	0.05439169
bta-miR-421	486.35	229.56	1.07182	0.00752368	0.05439169
bta-miR-95	177.04	56.47	1.46883	0.00764169	0.05439169
bta-miR-190a	308.17	134.42	1.23623	0.00870963	0.05895747
bta-miR-199a-5p	72.66	212.91	-1.45116	0.01011885	0.06678438
bta-let-7a-5p	99527.87	64910.6	0.616648	0.01232531	0.07213158
bta-let-7f	81592.87	49851.52	0.710595	0.01221875	0.07213158
bta-miR-126-5p	54104.54	31582.24	0.777001	0.01150292	0.07213158
bta-miR-185	1327.4	2402.35	-0.852611	0.01256838	0.07213158
bta-miR-200c	566.53	101.58	2.40995	0.0123985	0.07213158
bta-miR-2284x	1268.98	2115.9	-0.729719	0.01132033	0.07213158
bta-miR-374b	345.26	160.57	1.04547	0.01332768	0.07402714
bta-miR-432	86.84	225.32	-1.32715	0.01345948	0.07402714
bta-miR-1306	636.21	325.93	0.904917	0.01400314	0.07419623
bta-miR-98	2024.55	1161.18	0.791645	0.01405232	0.07419623
bta-miR-628	61.11	6.94	3.16643	0.01473826	0.07629216
bta-miR-150	3820.22	6240.45	-0.71107	0.01508768	0.07659898
bta-miR-23a	9331.98	14896.56	-0.674284	0.01564267	0.0779182
bta-miR-30c	1478.2	812.23	0.86938	0.01980585	0.09506808
bta-miR-423-5p	4281.42	7177.1	-0.742136	0.01967078	0.09506808
bta-miR-30b-5p	506.98	227.23	1.11121	0.02105387	0.09925395
bta-miR-25	9754.14	14904.35	-0.611267	0.02223328	0.10119974
bta-miR-455-5p	20.17	85.07	-2.27926	0.02191488	0.10119974
bta-miR-1307	1073.03	598.33	0.827786	0.02293889	0.1026418
bta-miR-147	14.29	69.54	-1.96006	0.02472405	0.10878584

bta-miR-374a	546.01	284.12	0.91637	0.02611783	0.11303456
bta-miR-454	623.56	342.92	0.856199	0.02825255	0.12030117
bta-miR-148a	5210.14	9922.88	-0.930946	0.03091561	0.12955112
bta-miR-2336	47.32	6.69	2.64842	0.03196711	0.13186433
bta-miR-151-3p	6502.7	4231.06	0.617622	0.03469661	0.14092163
bta-miR-15a	678.77	1091.3	-0.665954	0.04052165	0.16208659
bta-miR-204	237.65	112.08	1.03822	0.04170881	0.16434515
bta-miR-19a	321.23	157.87	0.930528	0.04574015	0.1775794
bta-miR-29b	1474.15	952.48	0.620024	0.05092075	0.19482722
bta-miR-155	3281.63	2320.77	0.502755	0.05659276	0.21343557
bta-miR-32	251.32	424.31	-0.725885	0.05895695	0.21922021
bta-miR-103	15841.71	11118.16	0.509666	0.06220604	0.22808883
bta-miR-10a	2111.03	1419.19	0.581321	0.06399668	0.23144006
bta-miR-27a-3p	1988.44	1248.74	0.661312	0.06807312	0.24285544
bta-miR-224	317.7	529.7	-0.791056	0.06999457	0.24638089
bta-miR-133b	0	21.18	-3.97026	0.07296038	0.24694539
bta-miR-455-3p	0	21.42	-3.97146	0.07296114	0.24694539
bta-miR-7859	23.51	0	3.70995	0.07240241	0.24694539
bta-miR-222	359.96	208.13	0.760387	0.07459019	0.24926343
bta-miR-23b-3p	4672.56	6680.35	-0.519383	0.07884009	0.2601723
bta-miR-205	2012.07	625.45	1.675	0.07995499	0.26059403
bta-miR-18a	220.94	121.59	0.875104	0.08144507	0.26221339
bta-miR-429	16.62	55.92	-1.69399	0.08250244	0.2624174
bta-let-7d	3716.76	2524.95	0.558712	0.08462274	0.26595718
bta-let-7b	42350.52	31970.63	0.405906	0.08677985	0.26952799
bta-miR-211	33.55	3.62	2.33074	0.08793305	0.26993401
bta-miR-125a	8347.64	5725.26	0.547211	0.09683466	0.28092694
bta-miR-2285aa	74.8	30.94	1.24985	0.09470349	0.28092694
bta-miR-2448-3p	96.18	44.81	1.23765	0.09488626	0.28092694
bta-miR-301a	310.1	186.96	0.740969	0.09410821	0.28092694
bta-miR-423-3p	2554.07	1809.63	0.494636	0.0958932	0.28092694
bta-let-7e	3360.19	2235.7	0.592254	0.10305481	0.29572251
bta-miR-379	240.47	378.77	-0.660478	0.10644234	0.29894444
bta-miR-744	178.88	90.41	1.00223	0.10601696	0.29894444
bta-miR-92b	37.09	86.3	-1.29112	0.10853975	0.30162625
bta-miR-221	6368.38	4526.74	0.49066	0.11150908	0.30402665
bta-miR-499	34.15	85.38	-1.4266	0.11170676	0.30402665
bta-miR-10b	6688.12	4787.26	0.48591	0.11564219	0.31152591
bta-miR-328	282.04	167.88	0.66159	0.12193997	0.32517325

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6. Acknowledgments

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