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TITLE:

Circulating microRNAs as biomarkers of various physiological processes underlying skeletal muscle growth in Piedmontese Cattle

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List of Abbreviations

MSTN	Myostatin
TGF-β	Transforming growth factor beta
MRFs	Myogenic regulatory factors
MyoD	Myogenic differentiation 1
MyoG	Myogenin
MYF5	Myogenic factor 5
MRF4	Myogenic regulatory factor 4
MEF 2	Myogenic enhancer factor 2
PAX 3	Paired box 3
PAX 7	Paired box 7
ActRII	activin type II receptor
MiRNAs	MicroRNAs
3'UTR	3'untranslated region
Ci-miRNAs	Circulating microRNAs
DGCR8	DiGeorge Syndrome Critical Region Gene 8
miRISC	miRNA-induced silencing complex
CXCL12	C-X-C Motif Chemokine Ligand 12
MVEs	Multi-vesicular endosomes
MVB	Multi- vesicular bodies
AGO2	Argonaute 2
HDL	High density lipoprotein
LDL	Low density lipoprotein
NPM1	Nucleophosmin 1
TLRs	Toll-like receptors
DE-miRNAs	Differentially expressed miRNAs
qRT-PCR	quantitative real-time PCR
EDTA	Ethylenediamine tetra acetic acid
ERNE	Extended Randomized Numerical aligner
KEGG	Kyoto encyclopaedia of genes and genomes
HPRT-1	Hypoxanthine phosphoribosyl transferase 1
IGF1R	Insulin-like growth factor 1 receptor
DMEM	Dulbecco's modified Eagle's medium
GM	Growth medium
DM	Differentiation medium
RPM	Reads per million
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PI3K/Akt/mTOR	phosphatidylinositol 3-kinase/alpha serine/threonine-protein kinase/mammalian target of rapamycin
PTEN	Phosphatase and tensin homolog
NFATc3	Nuclear factor of activated T-cells
MAFbx/atrogin 1	Muscle Atrophy F-box gene
MuRF1	Muscle RING-finger protein-1
RNAi	RNA interference

Declaration of Originality

I hereby declare that the work presented in this thesis and the thesis itself have been composed and originated by myself, unless otherwise specified. This work has not been submitted for any other degree or professional qualification.

The research described was carried out within a group and I made a substantial contribution to the work including data generation, analysis, presentation and interpretation. The included publications were included with permission from the remaining authors.

Rupal S Tewari

March 2022

Research Publications

- **Circulating skeletal muscle related microRNAs profile in Piedmontese cattle during different age.** Tewari RS, Ala U, Accornero P, Baratta M, Miretti S. Sci Rep. 2021 Aug 4;11(1):15815. doi: 10.1038/s41598-021-95137-w.PMID: 34349188

Conference Abstracts

- **Pilot study of circulating microRNAs profile in Piedmontese cattle during different growth periods**. Tewari RS, Baratta M, Miretti S. Proceeding book of International Congress on Domestic Animal Breeding Genetics and Husbandry. 11-13 September 2019 Prague, Czech. ISBN: 978-605-031-805-0. P82-85 (Oral)

- **Circulating microRNAs as biomarkers of various physiological processes underlying skeletal muscle growth in Piedmontese Cattle**. Tewari R., Miretti S., Barrata M. 'Non-Coding Genome' course. 25th Feb-4th March 2020, Institut Curie, Paris. (Poster)

- **Plasma expression profiling of circulating microRNAs in Piedmontese cattle during different periods of skeletal muscle growth**. Rupal S. Tewari, Ugo Ala, Elisabetta Macchi, Mario Baratta, Silvia Miretti. 74th SISVET Congress. 23-26 June 2021, Online platform. (Oral)

Abstract

Background The Piedmontese breed is known for a double-muscled phenotype. In recent studies, it has been reported that skeletal muscle can communicate with other tissues through the release of molecules in the circulating system. MicroRNAs (miRNAs) play an important role as regulators in physiological processes of skeletal muscle such as cell proliferation, differentiation, and hypertrophy. Emerging evidence shows that miRNAs are released from cells in the body fluids, called circulating miRNAs (ci-miRNAs).

Results Plasma samples of the Piedmontese cattle were collected at different ages: newborn (NB), 4-6 months (4-6M), 10-12 months (10-12M), and 15-17 months (15-17M). Small-RNA sequencing data analysis revealed the presence of 40% of muscle-related miRNAs among the list of top 25 highly expressed miRNAs and, 19 miRNAs showed differential expression too. Fourteen miRNAs involved in skeletal muscle physiology pathways were validated by qRT-PCR for expression analysis, 6 chosen from the differentially expressed miRNAs list and further 8 miRNAs arbitrarily selected based on literature and target prediction. Comparing NB with the other age groups, miR-10b, miR-126-5p, miR-143 and miR-146b were significantly up-regulated, whereas miR-21-5p, miR-221, miR-223 and miR-30b-5p were significantly down-regulated at different age points. Interestingly, qRT-PCR analysis disclosed a higher expression of miR-23a (one of the 8 arbitrarily selected miRNAs) in all the ages, though not differentially expressed among the groups. TargetScan algorithm predicted myostatin (MSTN) as the target gene for miR-23a and miR-126-5p. MSTN is a negative regulator of skeletal muscle hypertrophy, and we demonstrated the direct binding between miR-23a and miR-126-5p with 3'UTR of this gene. Further analysis revealed positive significant correlations between miR-126-5p and miR-146b and the weight of beef cattle for each age group (rho = 0.26; and rho = 0.36). On the other hand, miR-223 showed an inverse significant correlation with the weights (rho = -0.39).

Conclusion The knowledge about ci-miRNAs in bovine species is limited. These findings provide evidence on the presence of skeletal-muscle-related miRNAs in the plasma of beef cattle of different age groups. Furthermore, a direct binding has been shown between miR-23a and miR-126-5p and MSTN gene in bovine myoblast cells and the correlation between ci-miRNAs and body weight growth during different ages.

1. Background

This section includes a basic introduction to skeletal muscle hypertrophy feature in the Piedmontese cattle breed and a brief review of what is known about miRNA biogenesis and function as well as circulating miRNAs linked with physiological processes underlying skeletal muscle growth and hypertrophy.

1. 1 Piedmontese cattle

The Piedmontese cattle originate from the region of Piedmont located in northwest Italy. It is known for peculiar 'double-muscled' phenotype attributed to a higher increase in muscle mass relative to conventional cattle with age progression and for this reason, has been subjected to intensive genetic selection to gain mighty yields of high-quality meat (McPherron et al., 1997).

Historically, the Piedmontese breed is a cross between two distinct breeds, the Auroch and the Zebu. The Piedmont region constitutes the Northwest part of Italy and is bordered by the Italian Alps. Back in time, it was populated with primitive European cattle breed named Auroch (*Bos primigenius*) and *Bos taurus*. Several thousand years ago, Zebu (*Bos indicus*) a native breed of tropical areas started to migrate from India and Africa through Pakistan towards the Piedmont region preferring the Alpine vegetation. Due to aforesaid movement and new habitation of Zebu in the Piedmont region, over a period Auroch and Zebu cross-bred naturally and emerged as today's Piedmontese cattle breed (Italian Breeds of Cattle: Piedmontese).

The newborns are fawn-colored at the time of birth but turn into light grey upon maturity. The breed is of medium size. At birth, the calves weigh on average 40 to 45 kg, the cows around 550-600 kg and the male fattening calves become ready for slaughter at a weight of 550-680 kg. Usually, the weight gain is quite high, reaching above 2 kg a day in the calves between the weaning and slaughtering period under optimal feeding and housing conditions. The Piedmontese cattle are more proficient than normal cattle to convert feed into lean muscle and produce a higher percentage of meat (Casas et al., 1998). The dressing out percentage is very high too which is on an average 64-68% in the fattening calves, which peaks to 72% due to considerably fine bones and the low quantity of external fat. The conformation

of the carcass is excellent and always ranked S and E of the SEUROP classification (Nikolaou et al., 2020).

Figure 1.1 Piedmontese cattle breed (Picture courtesy: Animal facility, Dept. of Veterinary Science, University of Turin, Italy)

From the genetic point of view, the heritable 'double-muscle' phenotype in Piedmontese cattle is mainly due to skeletal muscle hypertrophy caused by the natural point mutation of the myostatin (MSTN) gene (Grobet et al., 1997). The Myostatin belongs to the transforming growth factor-b family (TGF-β) and is known to be one of the main regulators of skeletal muscle hypertrophy (Beyer et al., 2013; McPherron et al., 1997). In the Piedmontese, guanine is swapped with adenine at position 938 (G938A) of myostatin causing tyrosine insertion in place of cysteine and hampering the signaling portion of cystine-knot structural motif which is a characteristic of TGF-β family and hence inhibition of the biological activity of myostatin (Berry et al., 2002).

MSTN gene is highly conserved among mammalian species, and mutations in MSTN have been described in numerous species including sheep (Kijas et al., 2007), cattle (Grobet et al., 1997), and pig (Stinckens et al., 2008). A recent study showed the functional effect of miR-27b on bovine myostatin expression establishing the possible relationship between Piedmontese and double-muscled phenotype (Miretti et al., 2013).

1.2 Skeletal Muscle Hypertrophy

Skeletal muscle mass increases during postnatal development through a process of hypertrophy, i.e., enlargement of individual muscle fibre. It is a complex process where myoblasts proliferate to expand cell numbers and then fuse to form multinucleated myotubes that further undergo differentiation. New myofibers are formed during embryogenesis, but this process ceases around birth, and the number of myofibers remains fixed postnatally (Bonnet et al., 2010; White et al., 2010). During post-natal growth, an increase in the size of the elongating myofibers occurs by the fusion of myotubes with the pre-existing myofibers to increase the number of myonuclei per myofiber (hyperplasia of myonuclei) combined with increased net protein content (hypertrophy). During the animal's life, the phenomenon of hypertrophy slows down; however, low-level fusion of myoblasts into myofibers occur (Davis et al., 2002), and some low-level incorporation of differentiated myoblasts into myofibers has been reported in varying degrees (dependent on the muscle) in adults (Keefe et al., 2015). However, the extent of turnover or addition of myonuclei in normal mature myofibers is rather controversial. Since myonuclei of post-natal myofibers are post-mitotic, addition of any new myonuclei required for hypertrophy/ repair/ regeneration must be provided by

myoblasts generated from a pool of stimulated otherwise quiescent skeletal muscle stem cells (satellite cells) that are sandwiched between the plasmalemma of myofiber and basal lamina (Collins et al., 2005). In skeletal muscle tissue, these dynamic processes are actively regulated by several myogenic regulatory factors (MRFs) of the family of transcription factors such as MYOD1 (myogenic differentiation 1), MYF5 (myogenic factor 5), MYOG (myogenin) and MRF4 (myogenic regulatory factor 4) together with other transcription factors involved in myogenesis such as PAX3 (paired box 3), PAX7 (paired box 7) and the MEF2 (myocyte enhancer factor 2) family (Brand-Saberi, 2005). As shown in figure 1.2, MYOD and MYF5 actively take control of the first stage of skeletal muscle development by promoting the proliferation and differentiation of myogenic progenitor cells into myoblasts. During skeletal muscle growth, the level of MYOD and MRF4 goes down, leaving only MYF5 expressed in quiescent satellite cells in adults. MRFs are again redeployed during regenerative myogenesis to initiate and control phases of myogenic progression in satellite cells. MYF5 is rapidly upregulated and MYOD expression is initiated upon satellite cell activation, confirming the presence of both MYF5 and MYOD while satellite cells undergo their first cell division, and both are independently regulated during the cell cycle as satellite cells proliferation. MYOG initiates entry into myogenic differentiation, and MRF4 takes control later after the fusion (Zammit et al., 2017). Apart from MRFs various signaling pathways are also the in-charge of initiation of myogenic activities and maintenance of adult skeletal muscle mass by balancing muscle protein synthesis and degradation. The insulin-like growth factor-1 (IGF-1)/Akt (protein kinase B) pathways are positive regulators of skeletal muscle growth. In contrast, myostatin, NF-κβ, and glucocorticoid signaling negatively regulate skeletal muscle mass (Sandri et al., 2008). IGF1 is a potent growth factor and induces muscle hypertrophy by binding to a specific receptor (IGF1R) and activates phosphatidylinositol-3-kinase (PI3K). Activated PI3K produces phosphatidylinositol- 3,4,5, triphosphates (PIP3) and induces activation of the Akt protein. Eventually, Akt activates the mammalian target of rapamycin (mTOR) that controls protein synthesis (Rommel et al., 2001). Additionally, Akt inhibits the nuclear translocation of the FoxO family of transcription factors. Since, FoxOs play a key role in the regulation of muscle atrophy-related genes, MuRF1, and Atrogin1, inactivation of FoxOs prevents muscle protein degradation (Sandri et al., 2004). Myostatin and activin A are members of TGF-β superfamily and act as negative regulators of muscle mass by binding to

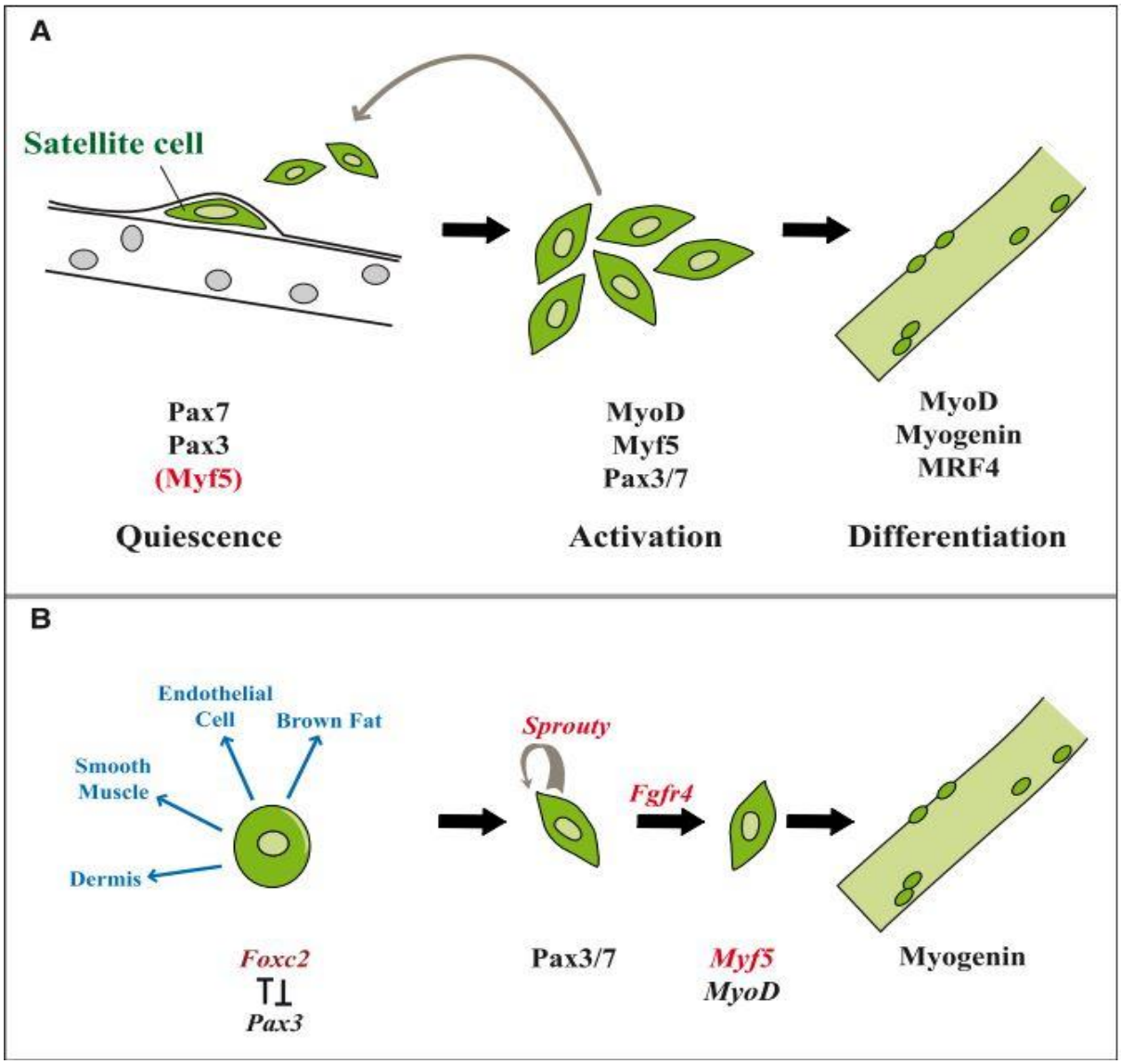


Figure 1.2 Activation and differentiation of muscle progenitor cells under the influence of myogenic regulatory factors (MRFs). The figure has been taken from Buckingham et al., 2014. (A) A series of steps starting from the activation of quiescent satellite cells to attain the differentiated state. (B) The movement of somatic cells undergoing myogenic changes shows how Pax3 activates target genes that regulate different stages of this process. Pax3 target genes are shown in red (Buckingham et al., 2014)

the Activin type II receptor (ActRII) (Schiaffino et al., 2021). Alongside MRFs and signaling molecules, miRNAs exert their effect on the myogenic pathways either in a specific or non-specific manner (Lagos-Quintana et al., 2002; Lee et al., 2001). For example, miR-27b promotes skeletal muscle cell proliferation in sheep and muscle hypertrophic phenotype in Piedmontese cattle by targeting MSTN gene (Miretti et al., 2013; Zhang et al., 2018), miR-21

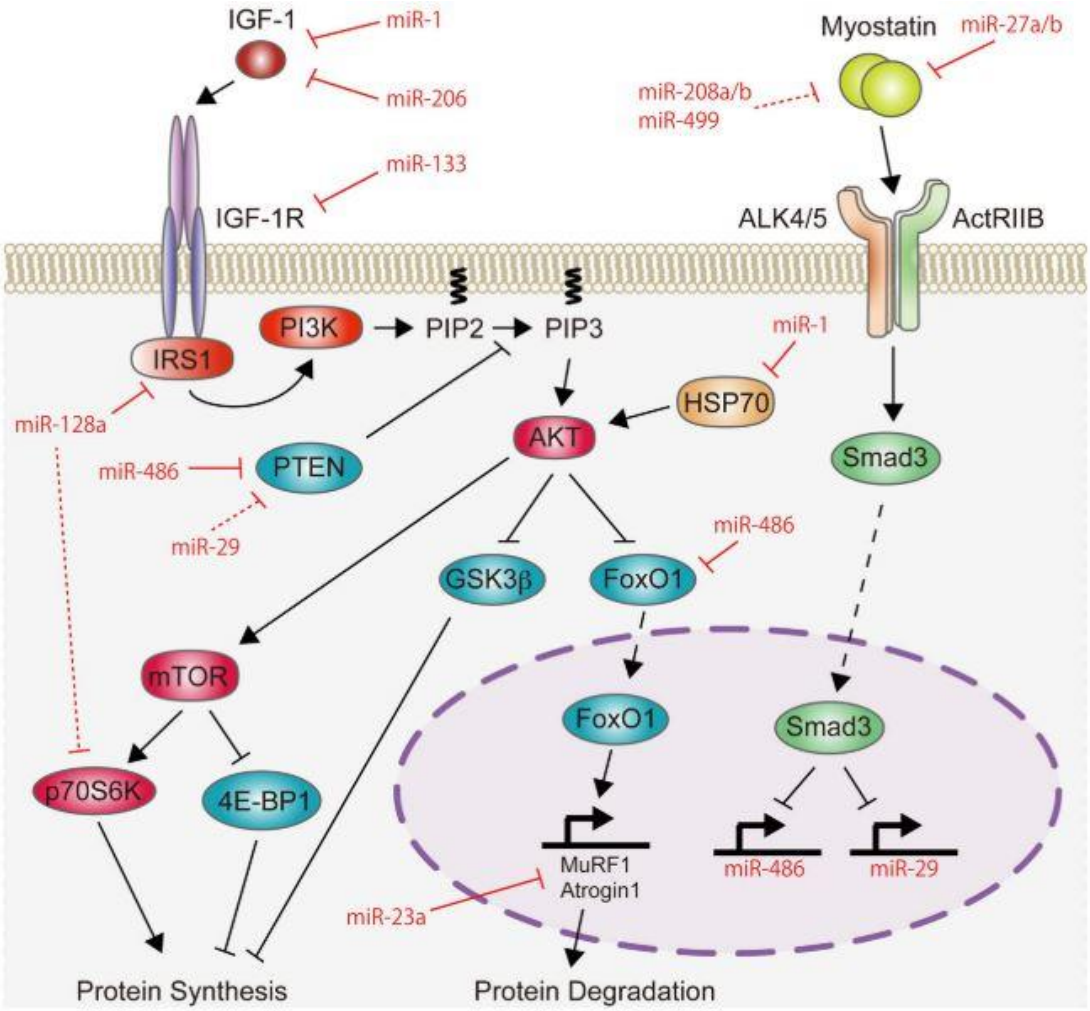


Figure 1.3 Illustrative diagram of miRNAs involved in the regulation of the IGF-1/Akt pathway and myostatin signaling. The figure has been taken from Hitachi et al., 2014. The activity of Akt protein is facilitated by IGF-1 and the activated Akt stimulates protein synthesis via mTOR. Akt as well inhibits protein degradation by suppressing GSK3β and FOXO1. miR-1, miR-133, miR-206, and miR-128a negatively regulate the IGF-1/Akt pathway by targeting positive regulators of the IGF-1/Akt pathway (IGF-1, IGF-1R, IRS1, HSP70, or p70S6K), while miR-29, miR-486, and miR-23a positively regulate this pathway by targeting negative regulators (PTEN, FoxO1, MuRF1, or Atrogin1). Whereas myostatin signaling is a negative regulator of skeletal muscle mass and myostatin expression is regulated by miR-27a/b, miR-208a/b, and miR-499. Red lines indicate the inhibitory function of miRNAs identified in skeletal muscle, while red-dashed lines represent the inhibitory function of miRNAs identified in cardiac muscle and cultured cells (Hitachi & Tsuchida, 2014)

regulates PI3K/Akt/mTOR signaling by Targeting TGFβI gene during skeletal muscle development in Pigs (Bai et al., 2015). Muscle-specific miRNAs (myomiRs), such as miR-1, miR-133a/b, and miR-206, were shown to be involved in the regulation of skeletal muscle hypertrophy by modulating the IGF-1–Akt pathway and myostatin signaling pathway (Hitachi et al., 2014; Huang et al., 2011; Kukreti et al., 2013). The role of miRNAs on the IGF-1/Akt pathway and myostatin signaling is summarized in figure 1.3

1.3 MicroRNAs (miRNAs)

1.3.1 What are miRNAs? The first miRNAs ever discovered were lin-4 and let-7, in *Caenorhabditis elegans*, in 1993 and 2000, respectively (Almeida et al., 2011). Since then, many studies have focused on understanding their evolution, biogenesis, and function in organisms. MiRNAs are a class of small 22-25 nucleotides (nt) endogenous non-coding RNAs that regulate gene expression at post transcription level by binding to 3'untranslated region (3'UTR) of target mRNAs thereby affecting targeted mRNAs stability and protein synthesis (Bartel et al., 2004). They are expressed in all eukaryotic cell types and are highly conserved among different species. In humans, endogenous miRNAs regulate at least 30% of genes and, thus, coordinate key cellular processes including proliferation, DNA repair, differentiation, metabolism, and apoptosis (Croce et al., 2005). Every miRNA has a unique nucleotide sequence and unique expression pattern depending on the cell type (Landgraf et al., 2007).

1.3.2. Biogenesis of miRNAs and mode of action

All miRNAs initially originate in the cell nucleus as long primary miRNAs (pri-miRNAs) transcripts containing 5'cap and a 3'polyA tail. The pri-miRNAs are then cut into∼70 nt hairpin precursor miRNAs (pre-miRNAs) by a microprocessor complex consisting of Drosha and DGCR8 proteins (Landthaler et al., 2004; Y. Lee et al., 2003). Further, pre-miRNAs are actively transported into the cytoplasm by Exportin protein and when in the cytoplasm, the pre-miRNA undergoes another cleavage step after being recognised by Dicer, an RNase III endonuclease (Figure 1.4). Dicer cleaves the pre-miRNA to produce a 22-nt duplex with 3' overhangs and 5' phosphates. It has been suggested that miRNA strand loading onto the miRNA-induced silencing complex (miRISC) depends on thermodynamic stability (Bartel et al., 2004). Most miRNAs remain in the cytoplasm after miRISC assembly, however, a few are

known to relocate back to the nucleus where they are assumed to have regulatory functions on transcription, for example binding to gene promoters (Krol et al., 2010; Winter et al., 2009).

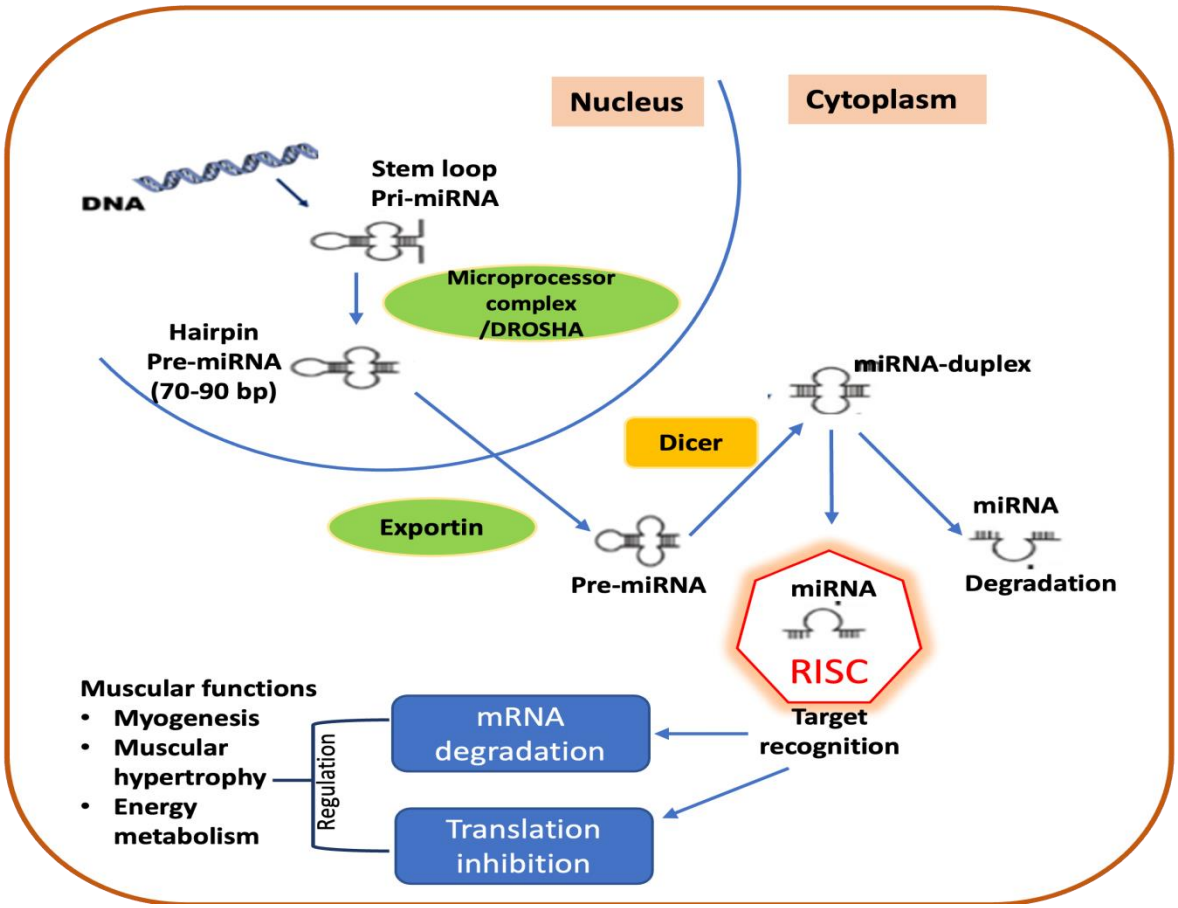


Figure 1.4 miRNA biogenesis pathway, miRNAs are transcribed in the nucleus by RNA polymerase II as primary miRNAs (pri-miRNAs). They are then processed into premature miRNAs (pre-miRNAs) by the microprocessor complex, followed by export to the cytoplasm by Exportin-5. In the cytoplasm, Dicer cleaves the pre-miRNAs into mature miRNA duplexes. The guide strand of the miRNA duplex incorporates into the miRNA-induced silencing complex (RISC). Finally, the mature miRNA targets mRNA transcript and binds through partial complementarity resulting in mRNA degradation or translation inhibition.

Once the miRISC is formed, miRNAs are available to base-pair with target mRNAs, resulting in endonucleolytic cleavage, deadenylation and translational repression (Huntzinger et al., 2011; Krol et al., 2010). miRNAs target specific mRNAs, primarily dictated by the conserved

miRNA seed sequence located at the 5’ end of the mature miRNA (position 2-7), and a large part of miRNAs are expressed in a tissue and developmental stage-specific manner (Coutinho et al., 2007; Jin et al., 2009). In addition to the seed sequence, their conserved 3’ end (positions 13-16) also contributes to miRNA base-pairing to targets. Targeting occurs primarily on the 3’ UTR of the mRNA, and less efficiently on its 5’ UTR (Bartel et al., 2009).

miRNAs which base pair perfectly to their target induce endonucleolytic cleavage, cutting approximately 10-11 nt from the 5’ end of the miRNA. However, in animals, perfect complementarity of miRNAs is rare; therefore, direct endonucleolytic cleavage is unlikely to take place and to have biological relevance (Bartel et al., 2004). Studies using transfected miRNA-targeted constructs in HeLa cells suggest that translational repression occurs before, and independently of, mRNA degradation, and materializes through both 1) reduction in initiation of translation and 2) a reduction in post-initiation translation (Meijer et al., 2013; Yi et al., 2008). Further studies in mouse Krebs-2 ascites cells support that miRNAs first block initiation of translation, prior to any mRNA degradation events (Mathonnet et al., 2007).

However, other research suggests that mRNA degradation driven by deadenylation is the main miRNA silencing mechanism. A recent study proposed that animal miRNAs lead only to mRNA deadenylation, which can result in translational repression or mRNA degradation depending on the stage of development of a given organism (Subtelny et al., 2014). Supporting this idea, a study on HeLa cells and mouse neutrophils focusing on miR-1, miR-155 and miR-223 shows that the majority miRNA gene silencing (at least 84 %) is attributable to mRNA destabilization (Guo et al., 2010) and an additional study supports the notion that miRNA actions are primarily deadenylation-driven (Chen et al., 2009).

Moreover, miRNAs regulate overlapping targets through complex networks depending upon base pair complementarity. An individual miRNA can fine-tune the expression of one or multiple targets and similarly a target can also be regulated simultaneously by multiple miRNAs (Bartel et al., 2009; Kim et al., 2013). This feature is either due to perfect bonding between miRNA–mRNA by Watson–Crick pairing in the miRNA 5’ proximal seed region (usually positions 2–8) or imperfect complementarity in the central part of the miRNA (usually positions 10 and 11) that prevents the RNAi-like endonucleolytic cleavage of the target mRNA in the middle of the duplex. Although one perfectly complementary site is sufficient for the

miRNA-induced cleavage of mRNA, but multiple imperfect sites recognized by the same or several different miRNAs can also cause translation repression but the molecular basis of it is still undetermined (Filipowicz et al., 2005; Zamore et al., 2005)

1.3.3. Circulating microRNAs

The following section focuses on circulating miRNAs (ci-miRNAs) and their carriers that grant stability to ci-miRNAs in circulation and body fluids and are summarised in Figure 1.5.

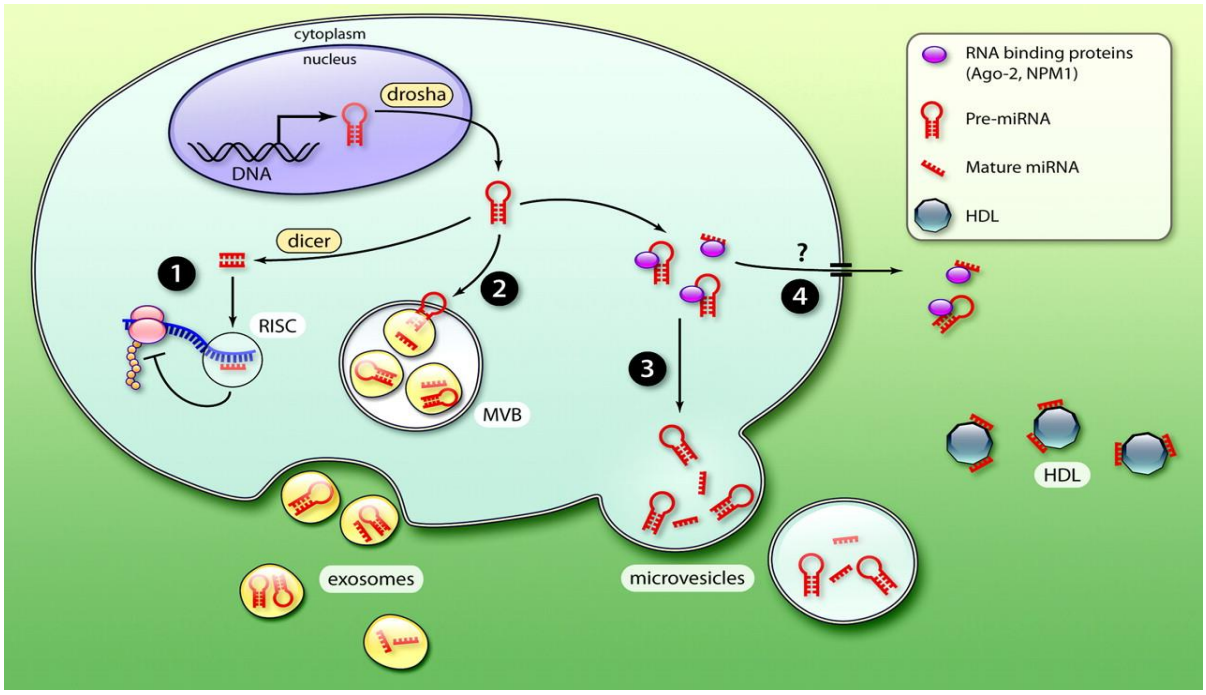


Figure 1.5 Mechanisms of transport of circulating microRNAs. Once generated, (1) miRNAs can remain in the cell where they target mRNA transcripts incorporated into RNA-induced silencing complex (RISC). (2) Alternatively, they can be released into the extracellular space enclosed inside exosomes, which form when multi-vesicular bodies (MVB) fuse with the plasma membrane. (3) Another mode of release into the extracellular matrix is inclusion in microvesicles, which form through budding from the plasma membrane. (4) Finally, HDL bound vesicle-free miRNAs can also be found in the circulation. (Creemers et al., 2012)

miRNAs can be released from cells through active secretion or passively via membrane leaking and circulate in the bloodstream or other body fluids in a stable cell-free form (Stoorvogel et al., 2012). Several accumulated research reports claimed that mature miRNAs are also present in a cell-free form in blood plasma and serum (Chen et al., 2008a; Chim et al., 2008; Mitchell et al., 2008). Indeed, the existence of extracellular ci-miRNAs in all other biological

fluids was confirmed later (Hanke et al., 2010; Kosaka et al., 2010; Weber et al., 2010). Although, the main mechanism that kept the miRNAs protected from nucleases outside the nucleus remained unclear for a long period of time; however, it was being already validated that the miRNAs were exported in culture encapsulated in exosomes (Valadi et al., 2007). The theory that extracellular miRNA is protected by encapsulation into membrane-vesicles emerged after miRNAs were detected miRNAs in peripheral blood (Hunter et al., 2008). Based on previous theories, it could be stated that miRNAs find their way into the extracellular space through multiple pathways. Early work into the origin of extracellular miRNAs identified miRNAs inside cellular vesicles, namely apoptotic bodies (800 nm – 5,000 nm), microvesicles (100 nm – 1,000 nm) and nanovesicles (30 nm – 100 nm) (Valadi et al., 2007; Zernecke et al., 2009a). The latter are also known as exosomes. Microvesicles are derived from simple budding of the cell membrane, while exosomes originate from multi-vesicular endosomes (MVEs) which are part of the endocytic pathway. MVEs eventually merge with the cell membrane and are released extracellularly; this appears to be an active, ATP-dependent mechanism (Raposo et al., 2013). Studies conducted later also identified miRNAs bound to several proteins in ribonucleoprotein complexes, outside of cellular vesicles (Wang et al., 2010). Argonaute 2 (AGO2), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and nucleophosmin 1 (NPM1) are examples of proteins that carry miRNAs in the extracellular space (Turchinovich et al., 2011; Vickers et al., 2011). Finally, miRNAs can be released passively because of cell lysis (e.g., hemolysis) (Kirschner et al., 2011).

An exciting fact is that foreign or endogenous miRNAs in circulation could potentially be taken up by other cell types and regulate targets within those cells, acting similarly to hormones. Indeed, reports suggest that extracellular vesicle-bound miRNAs can be taken up by cells through membrane fusion or via the endocytosis pathway (Janas et al., 2015). Another potential mechanism through which vesicle-contained circulating miRNA could affect gene expression is by binding cell membrane receptors in neighbouring cells, as shown for Toll-like receptors (TLRs) (Fabbri et al., 2012).

There appear to be multiple pathways allowing extracellular miRNAs to be taken up by cells however research is ongoing in this field. Studies with both vesicle and protein-associated miRNAs have shown that miRNAs that are taken up by recipient cells can have a quantifiable effect on recipient cell mRNA transcripts. Specifically, miRNAs are delivered via exosomes

from macrophages to endothelial cells, where they can target reporter constructs (Squadrito et al., 2014). In addition, a study showed that miR-126 secreted in apoptotic bodies from endothelial cells could indirectly modulate CXCL12 (C-X-C Motif Chemokine Ligand 12) levels in vascular cells (Zernecke et al., 2009b). Moreover, evidence is that not only vesicle-enclosed miRNAs have functional effects on recipient cells, HDL-bound miRNAs were also shown to be capable of down-regulating mRNA targets in hepatocytes (Vickers et al., 2011). However, despite multiple reports of miRNA-mediated mRNA modulation in recipient cells, it remains unclear if these effects are significant *in vivo*. In a study estimated that the median concentration of all miRNAs in human plasma was found in the range of 63 – 250 fM for normal subjects (Williams et al., 2013). However individual miRNAs would only be found at a fraction of these concentrations, and it remains to be shown if such amounts of miRNAs could have a quantifiable effect on distant cells systemically *in vivo*.

1.3.3 miRNAs involved in skeletal muscle physiology

Recent studies have shown several miRNAs to be involved in the regulation of myogenesis and differentiation of skeletal muscle tissue in a multitude of species (Lee et al., 2017a; Liu et al., 2019; Sheng et al., 2016). MicroRNAs which are exclusively or preferentially expressed in muscle are called myomiRs (Boutz et al., 2007; McCarthy et al., 2008; McCarthy et al., 2007) and the data suggests that currently eight miRNAs: miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486 and miR-499 are included in this category (McCarthy et al., 2008; Small et al., 2010; Rooij et al., 2008, 2009). MyomiRs are expressed in both cardiac and skeletal muscle except for miR-206, which is skeletal muscle-specific, and miR-208a, which is cardiac muscle-specific. miR-1, miR-133a/b and miR-206 have also been investigated in cattle (Chen et al., 2006; Dai et al., 2016; Hak et al., 2006). Some studies have provided evidence that not all myomiRs are solely expressed in a muscle-specific manner but may be detected in low levels in other tissues; however, their main function is still confined to muscle tissue (Lagos-Quintana et al., 2002; Walden et al., 2009). Based on the affinity of individual miRNAs toward specific tissues, they can be categorized as follows: (1) “tissue-specific” miRNAs with expression level more than 20-fold with respect to the mean values of other tissues, while (2) “tissue-enriched” miRNAs include miRNAs whose expression levels are lower than 20-fold in comparison with the mean values of other tissues, and (3) “undetectable” miRNAs are those

which are under the detectable limit (Lee et al., 2017a). An increasing number of studies have also identified other miRNAs involved in the post-natal skeletal muscle physiology and shed light on their mechanisms (Bai et al., 2015; Hitachi et al., 2014). Particularly, miR-27b (Miretti et al., 2013), miR-26a (Lee et al., 2015), miR-23a (Mercatelli et al., 2017), miR-143 (Zhang et al., 2017), miR-126-5p (Zuo et al., 2015), miR-146b (Khanna et al., 2014), miR-155 (Seok et al., 2011; Zhao et al., 2012) have emerged as regulators during skeletal muscle cell proliferation, differentiation and hypertrophy. Table 1.1 shows the list of some of the miRNAs that have been studied and found to be involved in skeletal muscle regulatory mechanisms. For instance, Wei Zhang and colleagues in 2018 demonstrated that miR-27b promotes skeletal muscle satellite cells proliferation by targeting myostatin gene in sheep (Zhang et al., 2018). In another study, a comparative analysis between miRNAs expressed in skeletal muscle tissue of Large White and Mini Pigs revealed microRNAs associated with postnatal muscle hypertrophy (Sheng et al., 2016). The crucial age points, in which the main expression differences were reported, were 60 and 120 days after birth, recognized as important stages for skeletal muscle hypertrophy and intramuscular fat deposition. A total of 263 miRNAs were significantly differentially expressed between the two breeds. Notably, ssc-miR-204 was significantly more highly expressed in Mini pig skeletal muscle at all postnatal stages compared with its expression in Large White pig skeletal muscle. Based on gene ontology and KEGG pathway analyses of its predicted target genes, it was concluded that ssc-miR-204 may exert an impact on postnatal hypertrophy of skeletal muscle by regulating myoblast proliferation.

Table 1.1 List of miRNAs involved in skeletal muscle physiology The table 'Confirmed and predicted targets' includes suggested and/or validated targets for each miRNA as reported in the cited studies. 'Function' lists the processes which are thought to be regulated by each miRNA according to the cited study. 'Species' indicates the species in which each study was conducted

miRNA	Confirmed and predicted targets	Function	Species	Study
miR-1	HDAC4	Myogenic differentiation	Goat	(Sui et al., 2020)

miR-133a/b	SRF	Skeletal muscle cells proliferation	Mice	(Chen et al., 2006)
miR-206	MSTN	Promotes muscle differentiation	Sheep	(Clop et al., 2006)
miR-27b	MSTN	Promotes skeletal muscle cell proliferation	Sheep Bovine	(Zhang et al., 2018) (Miretti et al., 2013)
miR-26a	Smad1 and Smad4	Promotes differentiation of myoblasts	Mice	(Dey et al., 2012)
miR-23a	MDFIC	Myogenic differentiation	Bovine	(Hu et al., 2020)
miR-143	IGFBP5 MYH7	Regulates proliferation and differentiation of skeletal muscle satellite cells Regulates muscle fiber differentiation	Bovine Pig	(Zhang et al., 2017) (Zuo et al., 2015)
miR-146b	Smad4, Notch1, and Hmga2	Promotes myogenic differentiation	Mouse	(Khanna et al., 2014)
miR-660	ARHGEF12	Skeletal muscle differentiation	Mice	(Yue et al., 2017)
miR-155	MAPK14, FLNB, ZBTB18 and CDK5)	Skeletal muscle development	Chicken	(Xu et al., 2020)
miR-30a/b/c	Runx1, Smarcd2, Snai2, and Tnrc6a	Involved in the differentiation of myoblasts	Mice	(Guess et al., 2015)
miR-221	mTOR pathway	Promotes proliferation of skeletal muscle satellite cells and inhibits differentiation	Rabbit	(Liu et al., 2018)
miR-21	TGFβI KLF3	Regulates the proliferation and differentiation of skeletal muscle satellite cells	Pig Chicken	(Bai et al., 2015) (Zhang et al., 2021)

1.3.4 Ci-miRNAs as biomarkers

Because of their abundance in the extracellular fluids, miRNAs have been investigated as potential non-invasive biomarkers. Ci-miRNAs make good biomarker candidates because they regulate key biological processes (Ioannidis et al., 2018; Webb et al., 2020), and some also demonstrate tissue-specific expression (Guo et al., 2014; Siracusa et al., 2018). They are remarkably stable and relatively easy to sample and quantify (Chen et al., 2008b; Mitchell et al., 2008).

So far, more than 100 ci-miRNAs have been proposed as biomarkers of multiple types of human cancer (Hamam et al., 2017; Müller et al., 2020; Sohel et al., 2020). Ci-miRNAs have also been linked to non-neoplastic diseases such as liver disease, cardiovascular disease, muscle differentiation and atrophy, diabetes and neurodegenerative diseases, among others (Párrizas et al., 2016; Pirola et al., 2018; Tasca et al., 2016; Zhang et al., 2020). In addition to disease, ci-miRNAs have also been linked to physiological states such as intense exercise and pregnancy.

In contrast to the extensive literature on human ci-miRNA biomarkers, there is only a limited number of studies that investigated the utility of ci-miRNAs as biomarkers in livestock species. For instance, certain skeletal muscle and cell proliferation liked miRNAs were found to be differentially expressed during developmental phases of early-weaned Chongming white goats. Deep sequencing comparison of plasma mRNA and miRNA expression profiles showed 18 miRNAs and 373 genes to be differentially expressed in pre and post-weaning goats and specifically downregulated miRNAs, chi-miR-206 and chi-miR-133a/b are muscle development-related and the others associated with cell proliferation (Liao et al., 2019). Another research group assessed a long list of circulatory mediators released in response to an acute immune stress induction in fifteen stress challenged (intravenous bolus of lipopolysaccharide (LPS; 400 ng/kg) female lambs to identify candidate biomarkers that can be used for the selection of stress-resilient animals. Blood was collected at 0, 2, 4, and 6 h after LPS challenge and the temperature was also recorded frequently. Biomarker responses revealed that all sheep had a monophasic febrile response to LPS challenge and miRNA (miR-145, miR-233, and miR-1246) expression levels increased and remained elevated throughout the study suggestive of their role in the process of acute reactions (Naylor et al., 2020). The

expression profiling of ci-miRNAs related to oestrus cycle in Holstein Friesian heifers was investigated and the plasma levels of four miRNAs (let-7f, miR- 125b, miR-145 and miR-99a-5p) were identified to be increased (up to 2.2- fold, $P < 0.05$) during oestrus (Day 0) with respect to other stages of the cycle (Days 8 and 16) (Ioannidis et al., 2016). In another work, it was demonstrated that grazing affected muscle-related ci-miRNA levels (e.g., miR-451) probably related to muscle movement (Muroya et al., 2015). Taken together, these studies show that ci-miRNAs have the potential to be used as biomarkers in farm animals, similar to humans.

2. General aims of the project

In livestock, limited information about the expression and the role of miRNAs in body fluids are present and no information is available about skeletal muscle related plasma miRNAs expression pattern in beef cattle during different growing stages. Moreover, miRNAs in circulation can impart their effect on the neighbouring tissues through cell-to-cell communication (Siracusa et al., 2018). This hypothesis made another basis to define the aims for my study. Firstly, I focused to describe the expression of plasma miRNAs profiles previously identified as related to muscle tissue and further investigated if they may be influenced by age or body weight in Piedmontese cattle. The obtained information could be used in the future to identify a few skeletal muscle-related microRNAs as biomarkers or as one of the parameters for the genetic selection of meat purpose breeds. Following are the three main aims of my project.

1. Explore and define a panel of expression of ci-miRNAs in the plasma samples collected from Piedmontese cattle just after birth i.e., within the first week of life and during skeletal muscle growth until getting slaughtered at 15-17 months of age.

2. Identify which of these ci-miRNAs have gene targets involved in skeletal muscle hypertrophy.

3. Investigate the possible relationship between muscle-related ci-miRNAs and body weight.

3. Aim 1: Ci-miRNAs expression profile at different age

3.1 Material and methods

3.1.1 Ethics statement

Blood samples were collected from Piedmontese cattle housed in the Animal Facility of Dept. of Veterinary Science, University of Turin, Italy, with the authorization of the Ethical Animal Welfare Committee (Prot. No. 663).

3.1.2 Experimental design

A pilot study to determine the presence and, the differential expression of bovine plasma miRNAs was strategized as depicted in Figure 3.1-a. In brief, the target animals were divided into four groups: newborn (NB), 4-6 months old (4-6M), 10-12 months old (10-12M) and 15-17 months old (15-17M) corresponding to different phases of growth and muscular phenotype. All the outcomes during this study were measured in a cross-sectional way and not longitudinally. Plasma miRNAs of three animals for each age group were sequenced to identify a panel of detectable and/or differentially expressed (DE-miRNAs) ci-miRNAs at four-time points. The expression of candidate miRNAs using quantitative real-time PCR (qRT-PCR) was conducted using n=22, n=22, n=21, and n=20 animals of each age group, respectively. Body weight during the four age periods beginning from birth until getting slaughtered was collected.

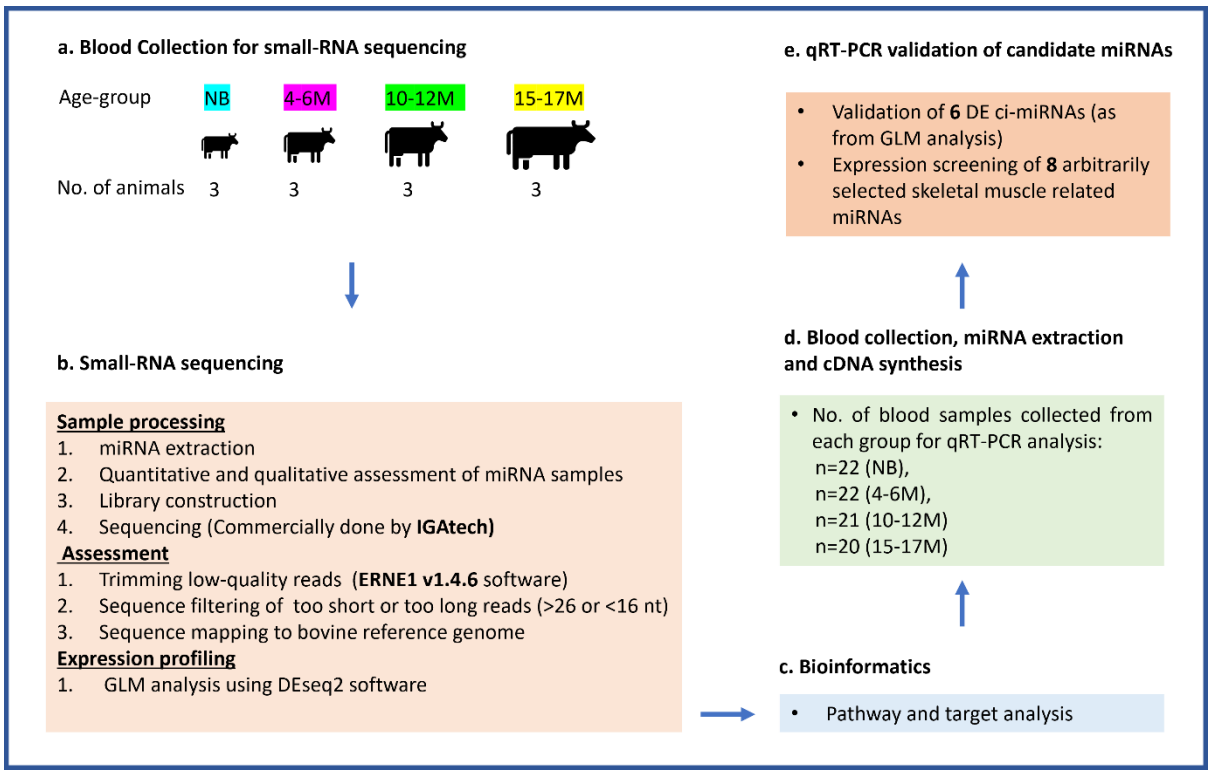


Figure 3.1 Schematic diagram of the study and workflow

a. Target animal population divided into 4 groups (NB, 4-6M, 10-12M and 15-17M). Blood sampling was carried out from total of 12 animals for Small RNA-Sequencing (3 per group). b. For small RNA-Seq, sample processing (1 and 2) was performed by me whereas the sample processing (3 and 4), assessment (1, 2, 3) and expression profiling (1) were commercially performed by IGAtech, Italy. c. Bioinformatic tools: TargetScan database was used to collect miRNA-mRNA target predictions to perform KEGG pathway analysis and specific target inspection. d. Blood collection from total of 85 animals, miRNAs extraction and cDNA synthesis. e. qRT-PCR expression analysis of 14 selected miRNAs.

3.1.3 Sample collection and processing

Blood sampling was carried out from total of 12 animals for Small RNA-Sequencing (3 per group) and total of 85 animals (blood samples were collected from 'n' number of animals falling into four age groups: n=22 samples/NB; n=22 samples/4-6 M; n=21 samples/10-12M; n=20 samples/15-17M) for qRT-PCR validation. Samples were collected in 10 mL K2 EDTA Vacutainer tubes (Becton Dickinson, NJ, USA) by jugular vein puncture, using 18G needles (Becton Dickinson, NJ, USA) and instantly stored in ice. Within two hours of collection,

samples were centrifuged at 1900 g for 10 min at 4°C to remove blood cells, followed by second centrifugation at 16000 g for 10 min at 4°C to remove cellular debris and platelets (Ioannidis et al., 2016). One aliquot of 500µl of each plasma sample was immediately used for RNA extraction and the remaining aliquots were stored at -80°C.

3.1.4 RNA extraction and cDNA synthesis

Total RNA was extracted using Maxwell RSC miRNA Plasma and Serum kit (Promega, Madison, USA) according to the manufacturer's protocol. Before RNA extraction from 500µl of plasma sample, 1µl of a UniSp2, 4, 5 miRCURY LNA spike-in (Qiagen, Maryland, USA) was spiked-in as an internal control. Quantification of miRNA was performed by Quantus 3.0 fluorometer (Invitrogen, Chicago, USA). Reverse transcription (RT) was performed using miRCURY LNA RT II Kit (Qiagen, Maryland, USA) according to the manufacturer's protocol. cDNA was synthesized from 0.8µl of total RNA. A quantity of 0.5 µl of a mix of Ce-miR39 and Unisp6 miRCURY LNA spike-in (Qiagen, Maryland, USA) was added as an internal control.

3.1.5 Library preparation and small RNA sequencing

'TruSeq SmallRNA Sample Prep kit' (Illumina, San Diego, USA) was used for library preparation following the manufacturer's instructions. RNA samples were previously quantified, and quality tested by Agilent 2100 Bioanalyzer RNA (Agilent technologies, Santa Clara, USA) or by Caliper RNA LabChip GX (Caliper Life Sciences, Hopkinton, USA). Final libraries were quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and quality tested by Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA) or by Caliper RNA LabChip GX (Caliper Life Sciences, Hopkinton, USA). Libraries were then prepared for sequencing and sequenced on single-end 75 bp mode on NextSeq500 (Illumina, San Diego, CA). The Bcl2Fastq 2.0.2 version of the Illumina pipeline was used to process raw data for both format conversion and de-multiplexing. Adapter sequences were masked with Cutadapt v1.11 from raw Fastq data using the following parameters: --anywhere (on both adapter sequences) --overlap 5 --times 2 --minimum-length 35 --mask-adapter. Lower quality bases and adapters were trimmed by ERNE v1.4.6 software. Above mentioned small RNA-Seq procedures were commercially performed by IGA Technology Services (IGAtech, Udine, Italy) (Fig 3.1-b).

3.1.6 Quantitative assessment of plasma miRNAs expression (qRT-PCR validation)

The qRT-PCR with total cDNA was performed using SYBR green II PCR Kit (Qiagen, Hilden, Germany). PCR amplifications were performed on Bio-Rad CFX Connect Real-Time System (Bio-Rad, Hercules, USA). The qRT-PCR parameters specific for miRNAs and gene expression are detailed in Table 3.2. The differential expression among miRNAs was carried out by comparing the normalized Cq values (ΔCq) for all biological replicates between the two groups of samples. Based on a stable number of reads obtained by sequencing and a stable value of qRT-PCR expression among the groups, miR-378 was selected as the reference gene. The fold change of expression of transcript/miRNA was calculated by the $2^{-\Delta\Delta Cq}$ method where ΔCq = Cq of the target gene/miRNA−Cq of the reference gene/miRNA (Miretti et al., 2017). Data were expressed as fold-change with respect to NB samples.

Primers were pre-designed and commercially synthesized by LNA technology (Qiagen, Maryland, USA) (Table 3.1).

Table 3.1: Primer assays used to detect bovine miRNAs expression through qRT-PCR

miRNA	**Primer sequence 5'-3'**
miR-126-5p	MIMAT0000444: 5'CAUUAUUACUUUUGGUACGCG
miR-10b	MIMAT0000254: 5'UACCCUGUAGAACCGAAUUUGUG
miR-143	MIMAT0009233: 5'UGAGAUGAAGCACUGUAGCUCG
miR-223	MIMAT0000280: 5'UGUCAGUUUGUCAAAUACCCCA
miR-30a-5p	MIMAT0003841: 5'UGUAAACAUCCUCGACUGGAAGCU
miR99a-5p	MIMAT0003537: 5'AACCCGUAGAUCCGAUCUUGU
miR-146b	MIMAT0005595: 5'UGAGAACUGAAUUCCAUAGGCUGU
miR-21-5p	MIMAT0003528: 5'UAGCUUAUCAGACUGAUGUUGACU
miR-221	MIMAT0003529: 5'AGCUACAUUGUCUGCUGGGUUU
miR-30b-5p	MIMAT0000420: 5'UGUAAACAUCCUACACUCAGCU
miR-23a	MIMAT0003827: 5'AUCACAUUGCCAGGGAUUUCCA
miR-155-5p	MIMAT0000646: 5'UUAAUGCUAAUCGUGAUAGGGGU
miR-660	MIMAT0004344: 5'UACCCAUUGCAUAUCGGAGCUG

miR-30c-5p	MIMAT0000244: 5'UGUAAACAUCCUACACUCUCAGC
miR-378	MIMAT0000732: 5'ACUGGACUUGGAGUCAGAAGGC

Table 3.2: qRT-PCR parameters for miRNA assay

Target to be quantified	**qRT-PCR parameters**	**Kit used**
miRNA expression	1. 95°C for 15 min 2. 94°C for 15 sec, slow ramp rate to 1.0 C/sec 3. 56°C for 30 sec, slow ramp rate to 1.0 C/sec 4. 70°C for 30 sec, slow ramp rate to 1.0 C/sec Repeat points 2-4 for 40 cycles 5. Melt curve 60°C to 95°C, increment of 0.5 C for 5 sec	SYBR Green PCR Kit (Qiagen, Hilden, Germany) compatible with cDNA made using miRCURY LNA RT II Kit (Qiagen, Maryland, USA)

3.1.7 Statistical analysis

The GLM analysis was performed cumulatively for all the four groups using DESeq software taking into consideration log2 fold change >±0.58 and $p < 0.05$ (Anders & Huber, 2010). qRT-PCR data was assessed on GraphPad Prism (9.0.2 version) by one-way ANOVA test and Student's t- test. Distribution normality assumption was assessed through the Shapiro Wilk test. Data were expressed as mean ± SEM. Differences were considered as significant at a level of $p < 0.05$. For the miRNAs pathway analysis and target gene prediction, bovine miRNAs were related to their miRNA family through TargetScan 7.2 association table (TargetScan 7.2 Data Download). miRNA-target genes were associated to our miRNAs of interest by selecting from TargetScan 7.2 predicted target genes with at least one conserved binding site and a cumulative weighted context++ scores (CWCS) > −0.05 (Lecchi et al., 2019) KEGG (Kyoto encyclopaedia of genes and genomes) enrichments and functional analysis were conducted through Bioconductor (Bioconductor - Home) package ClusterProfiler, version 3.12.0. A 0.05 cut-off value was chosen for both pValue and qValue and BH (Benjamini-Hochberg) and False

Discovery Rate control method for multiple testing was considered. Human functional annotations were based on org.Hs.eg.db packages. All analyses were run in R, a free software environment for statistical computing and graphics, release 3.6.3. (Agarwal et al., 2015; Yu et al., 2012).

3.2 Results

3.2.1 miRNAs sequencing data

Plasma miRNAs were sequenced to explore the panel of expression of ci-miRNAs in Piedmontese cattle across the four ages of growth and to identify DE-miRNAs among the different groups. Small-RNA libraries were prepared from individual plasma samples collected from total of 12 animals with 3 animals from each age group as shown in figure 3.1a. First, low-quality reads and adapter sequences were removed to obtain clean reads and then the clean data were mapped to the bovine reference genome (Ensembl, http://www.ensembl.org/info/genome/genebuild/ncrna.htm). In total, 237 *Bos taurus* annotated miRNAs (bta-miRNAs) were detected. The number of reads per million (RPM) obtained for each sample after removing reads with low quality (reads without an adapter, short reads and reads with multiple undetermined base calls) are provided in Table 3.3.

For each class of sequence size, a bar graph was plotted, whose height represents the abundance of the sequences with a length included in the considered class. The most common read lengths after removing the sequencing adaptors ranged between 20-23 nt, which corresponds to the length of mature miRNAs (Figure 3.2). The graph gives the first summary statistics on the size distribution of the small RNAs sequences produced by the sequencing run.

Table 3.3 Number of reads in million (RPM) for 3 samples per group Table shows the number of reads per million (RPM) obtained for each sample after removing low quality reads

Library	RPM
NB_A1	11.18
NB_A4	12.98
NB_A6	12.59

4-6_B3	16
4-6_B4	14.88
4-6_B5	6.85
10-12M_C1	12.23
10-12M_C2	11.25
10-12M_C3	9.7
15-17M_D2	4.75
15-17M_D4	7.03
15-17M_D5	4.7

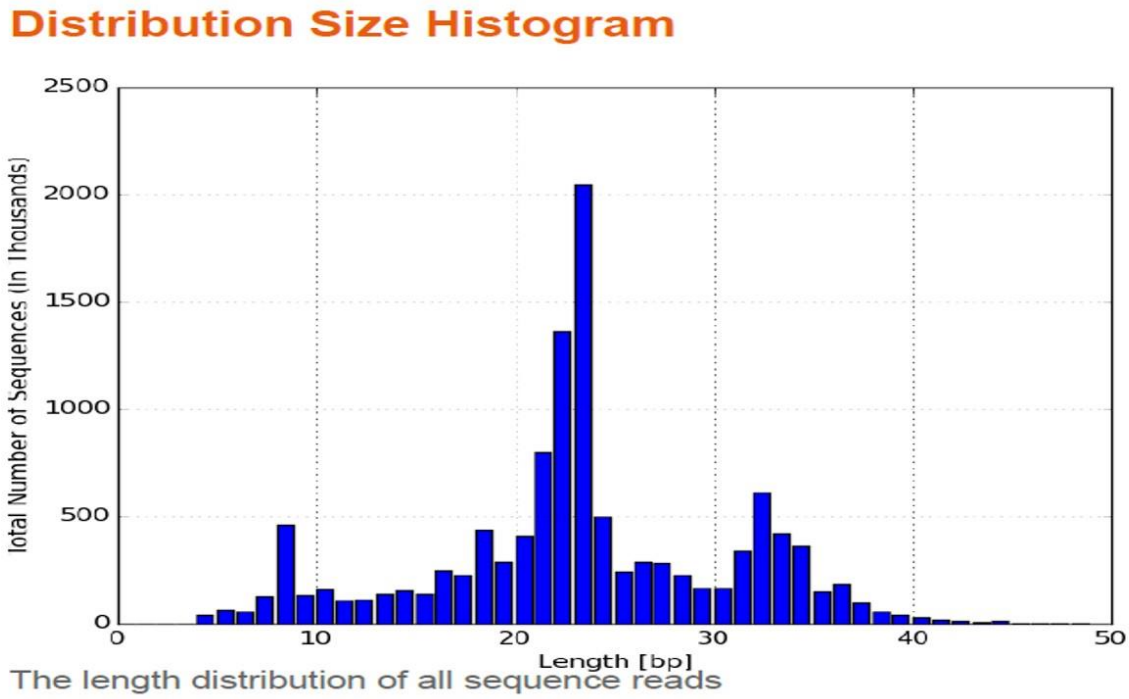


Figure 3.2 Size distribution plot. The plot and read length distributions showed a peak at 20–23 nt which is the main feature of mature miRNAs.

3.2.2 Circulating miRNAs profile in four age-groups of beef cattle

The number of miRNAs with a normalized expression between 0 and 100 RPM were 180, 181, 179 and 182 accounting for 75.9%, 75.9%, 75.5% and 76.7% respectively in NB, 4-6M, 10-12M and 15-17M groups. Likewise, the number of miRNAs with a normalized expression between 100 and 1000 RPM were 73, 70, 68 and 67 during the four age periods tested, accounting for

23.6%, 24.7%, 26.3% and 33.1% of the total mature miRNAs, respectively. There were 30, 32, 31 and 35 miRNAs with mean expression above 1000, accounting for 9.7%, 11%, 11.9% and 17.3% of the total mature miRNAs, respectively. Lastly, the number of miRNAs with mean expression over 10000 RPM at four stages were 4, 5, 4 and 6 respectively for each group, accounting for 2.7%, 2.9%, 3.1% and 4.1%, respectively (as shown in figure 3.3).

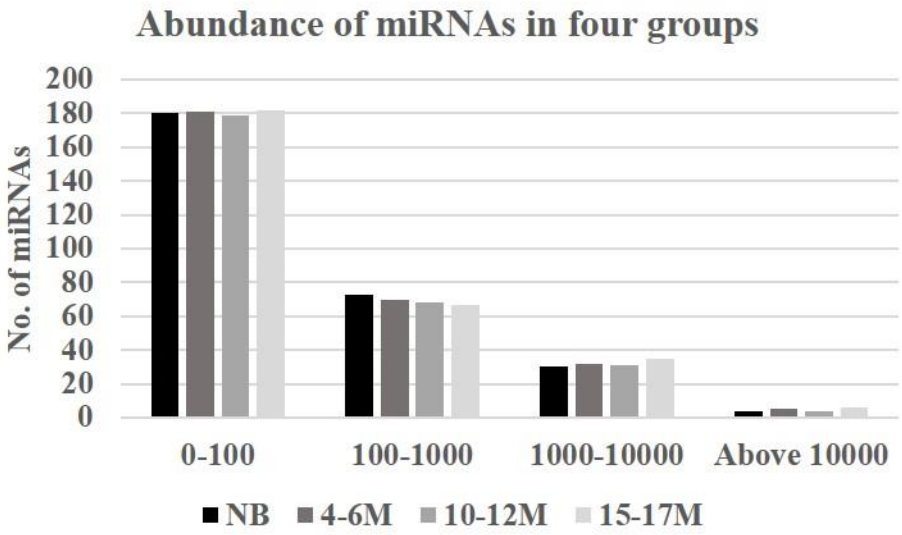


Figure 3.3: Abundance of miRNAs in four groups of animals with different ages, (NB=new-born; 4 to 6 months; 10 to 12 months; 15 to 17 months) miRNAs were counted based on the expression level (RPM) for each group (n=3).

Out of 100 most expressed genes in blood plasma samples, the list of top 25 expressed genes is reported in Table 2.4. Among the 25 highly expressed miRNAs, 10 (40%) were muscle-related miRNAs, including miR-486 (Small et al., 2010), miR-26a (Lee et al., 2015), miR-27b (Miretti et al., 2013), miR-30e-5p (Jia et al., 2017), miR-30a-5p (Guess et al., 2015), miR-146b (Khanna et al., 2014), miR-21-5p (Borja-Gonzalez et al., 2020), miR-660 (Yue et al., 2017), miR-186 (Antoniou et al., 2014), miR-126-5p (Jebessa et al., 2018). miRNAs highlighted in bold were validated by qRT-PCR for the expression analysis. The GLM analysis revealed 19 miRNAs to be differentially expressed (DE). Out of 19 DE, miRNAs numbered from 1 to 12 in table 2.5 were upregulated whereas the remaining 13 to 19 were significantly downregulated.

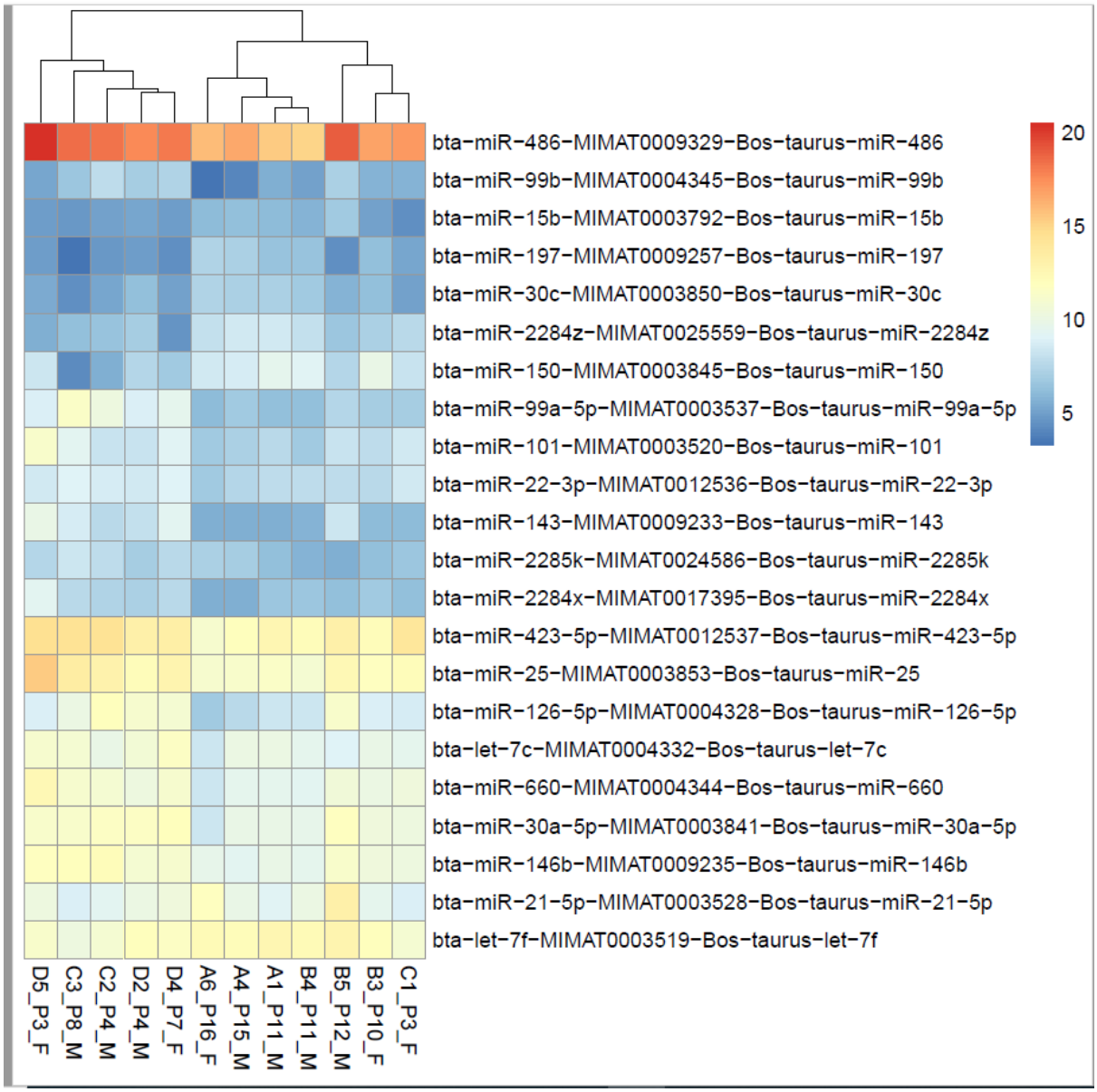


Figure 3.4 Most expressed miRNAs in the plasma samples using sequencing Heatmaps showing the 20 most expressed miRNAs in plasma. FDR < 0.05 for all miRNAs and three samples per group (n = 3). Samples A1, A4 and A6 belong to new-born, samples B3, B4, B5 are from 4-6 months old group, samples C1, C2, C3 are from 10-12 months old group and D2, D4, D5 belong to 15-17 months old animals' category.

Table 3.4: The 25 most expressed miRNAs in blood plasma samples. The expression of these 25 miRNAs is highest collectively in all the four age groups in terms of clean RPM without adaptors.

S. No.	miRNA	NB	4-6M	10-12M	15-17M
1.	miR-486	7031	237055	28245	651808
2	miR-191	32255	24868	25043	15215
3	miR-148a	17607	17534	20170	16982
4	miR-423-5p	4108	6225	20299	14600
5	miR-26a	12639	13707	6586	8398
6	miR-let-7i	8815	10288	5896	11991
7	miR-25	2469	3640	8086	18510
8	miR-27b	8152	6917	7385	6537
9	miR-30d	6567	5240	4035	3246
10	miR-let-7f	5450	5566	1747	3282
11	miR-92a	2297	3187	3617	3782
12	miR-151-3p	2347	2837	3121	2709
13	miR-30e-5p	2488	2539	2241	1747
14	**miR-30a-5p**	**727**	**1959**	**2294**	**3268**
15	**miR-146b**	**844**	**1521**	**3241**	**2589**
16	miR-let-7a-5p	2345	1909	1032	1739
17	**miR-21-5p**	**1681**	**3583**	**529**	**1204**
18	**miR-660**	**598**	**1088**	**1862**	**3056**
19	miR-186	1156	1893	2139	1360
20	miR-6529a	1625	1062	2119	1019
21	miR-26b	2198	1520	970	1106
22	miR-181a	1756	1590	746	1544
23	miR215	757	1739	2004	966
24	miR-let7c	799	741	1224	2341
25	miR-126-5p	202	1120	1809	1550

Table 3.5: Nineteen miRNAs were up or downregulated according to GLM analysis of sequencing data. The miRNAs numbered from 1 to 12 were upregulated and remaining miRNAs numbered from 13 to 19 were down regulated among the age groups subjected to Pair-wise and GLM analysis. Highlighted miRNAs in bold were selected for qRT-PCR validation.

S.No.	miRNAs	P value
1.	miR-let-7e	0.03
2.	miR-99b	0.01
3.	**miR-126-5p**	**0.0003**
4.	miR-10a	0.03
5.	miR-1468	0.01
6.	**miR-10b**	**0.01**
7.	**miR-30a-5p**	**0.0009**
8.	miR-15b	0.003
9.	miR-342	0.01
10.	**miR-143**	**0.00006**
11.	**miR-99a-5p**	**0.00006**
12.	miR-6119-5p	0.04
13.	miR-374b	0.02
14.	miR-150	0.003
15.	miR-1296	0.0004
16.	miR-1306	0.04
17.	**miR-223**	**0.02**
18.	miR-19b	0.03
19.	miR-197	0.0003

Out of the 19 DE miRNAs, six miRNAs were selected for qRT-PCR validation and are highlighted in bold in table 3.4. The selection was based on the differential expression in a sequenced plasma sample, higher number of reads, previous studies depicting their involvement in skeletal muscle development pathways and target gene analysis. Our previous study showed that skeletal muscle-specific miRNAs, namely miR-1, and miR-206 were abundant in the

muscle tissues of Piedmontese cattle (Miretti et al., 2011), whereas they were detected in the plasma profiles with a mean expression level less than 20 RPM, hence they were excluded for subsequent analysis (Ioannidis et al., 2018).

3.2.3 Pathway enrichment analysis (KEGG) of DE-miRNAs

To understand the potential functions of the DE miRNAs, TargetScan 7.2 database (as in the M&M section) was used to identify putative target transcripts. These genes were then analyzed to predict which specific molecular pathways showed possible participation in our biological setting. KEGG analysis showed statistically significant 65 pathways (figure 3.4). The topmost pathways that were found to be significantly enriched for the target genes were PI3K-Akt signaling pathway, MAPK, FOXO, mTOR, TGF-βsignalling pathways.

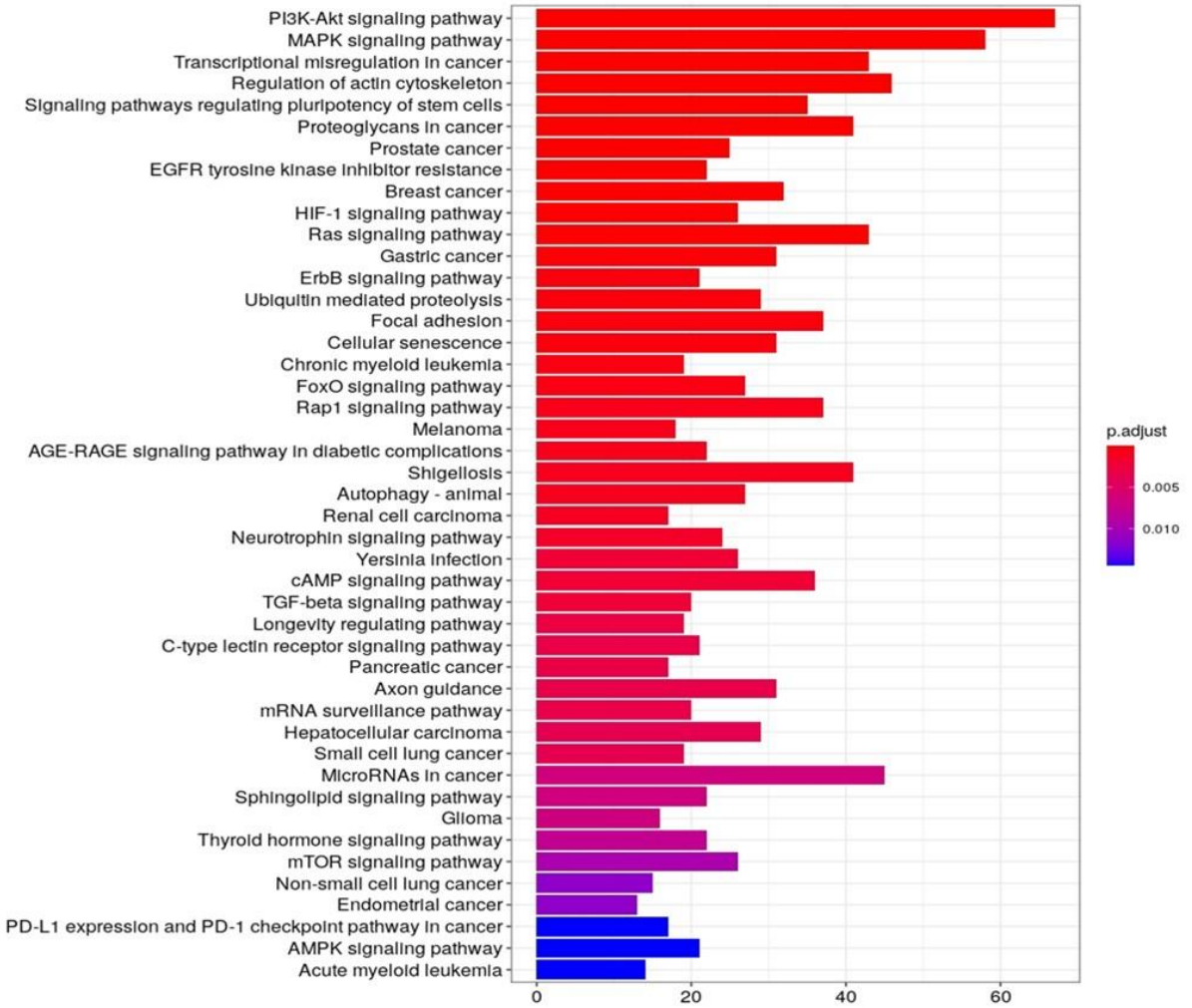


Figure 3.5 First 45 most significantly enriched KEGG pathways On the X-axis, a number of involved genes is reported, and the different colors (from red to blue) highlight different magnitude of the pValue.

The significantly enriched pathways were mainly involved in energy metabolism including PI3K-Akt and MAPK or regulating pluripotency of stem cells and skeletal muscle growth signaling pathways, including FoxO, TGF-β and mTOR signaling. These pathways are also known to be closely related to the regulation of myogenesis., such as MAPK signaling pathway (Lee et al., 2017b). The Wnt signaling pathway regulates the homeostasis of adult muscle (Maltzahn et al., 2012) and is involved in adipogenesis metabolism. For my project, I focused on the TGF-beta signaling pathway because many studies have reported that the TGF-beta family played important roles in skeletal muscle development (Acharjee et al., 2014; Kollias et al., 2008). Myostatin is a member of the TGF-beta superfamily and acts as a negative regulator of postnatal muscle growth (McPherron et al., 1997) and the IGF-1/Akt/mTOR pathway has been shown to be crucial in promoting muscle hypertrophy (Glass et al., 2003).

The putative target genes with cumulative context scores greater than -0.27 were shortlisted which might be regulated by the selected miRNAs. Some miRNAs targeted more than 1000 predicted targets, and some other target genes were putatively regulated by more than two miRNAs.

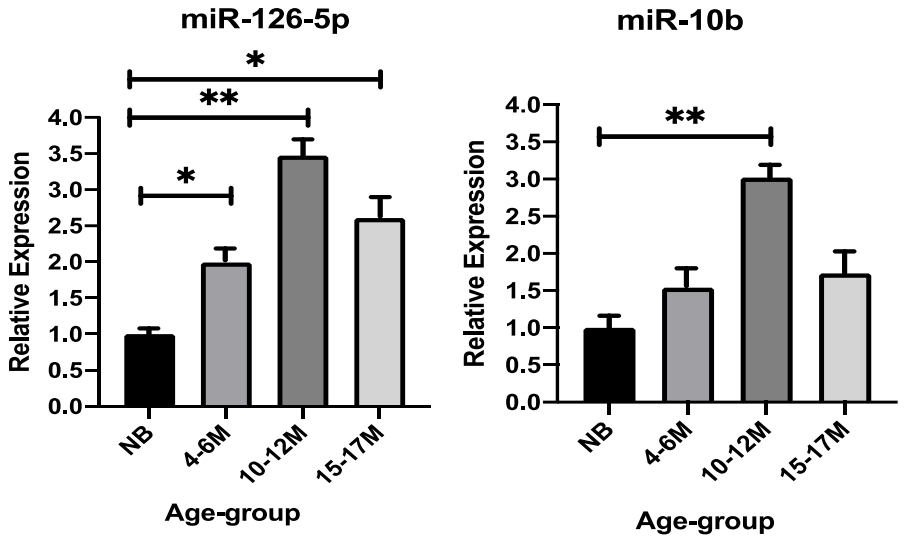
3.2.4 Age affects plasma miRNAs expression

To verify if age can affect the expression of selected miRNAs, I decided to further analyze some of the differences, in miRNAs level, identified with $P < 0.05$. For this, I performed qRT-PCR on plasma samples collected from total 85 animals. qRT-PCR is usually considered the 'gold standard' for validation of high-throughput analyses results due to its relative high accuracy, the ability to factor-in the amplification efficiency for each miRNA and the absence of severe biases which can typically alter miRNA abundance data obtained from sequencing analyses (Fuchs et al., 2015; Mestdagh et al., 2014).

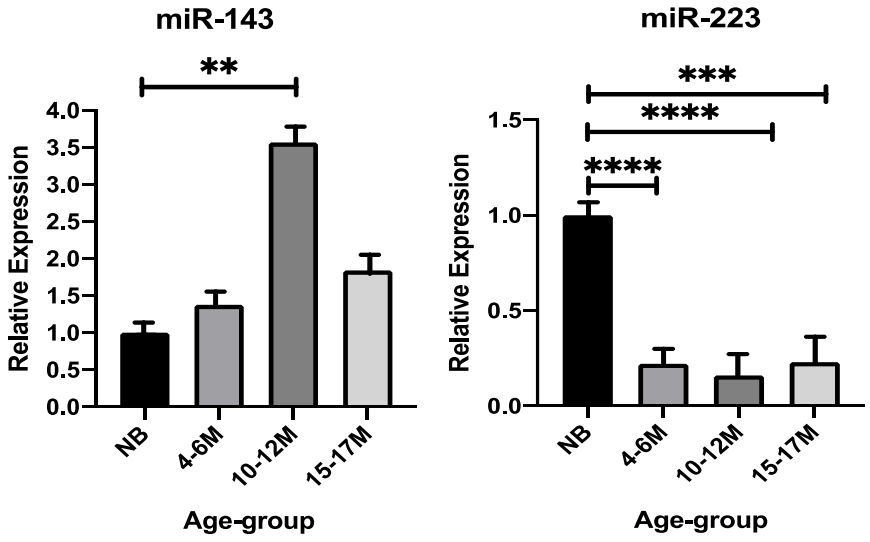
Respectively 6 miRNAs (miR-99a-5p, miR-143, miR-126-5p, miR-30a-5p, miR10b and miR-223) among the sequenced DE-miRNAs were selected for validation.

Additionally, 8 miRNAs (miR-146b, miR-21-5p, miR-221, miR-30b-5p, miR23a, miR-155-5p, miR-660 and miR-30c-5p) were arbitrarily chosen from the complete list of sequenced miRNAs combining literature knowledge, a number of reads (>20 RPM), target genes prediction (TargetScan version 7.2) and KEGG pathway analysis. This was adopted as a complementary approach to identify biologically relevant miRNAs which may have been missed by high-throughput analyses.

a.



b.



c.

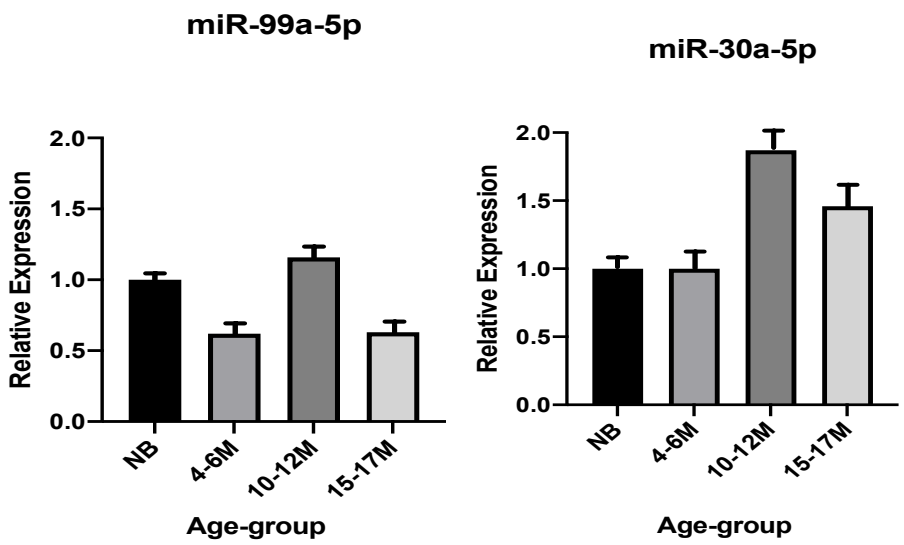
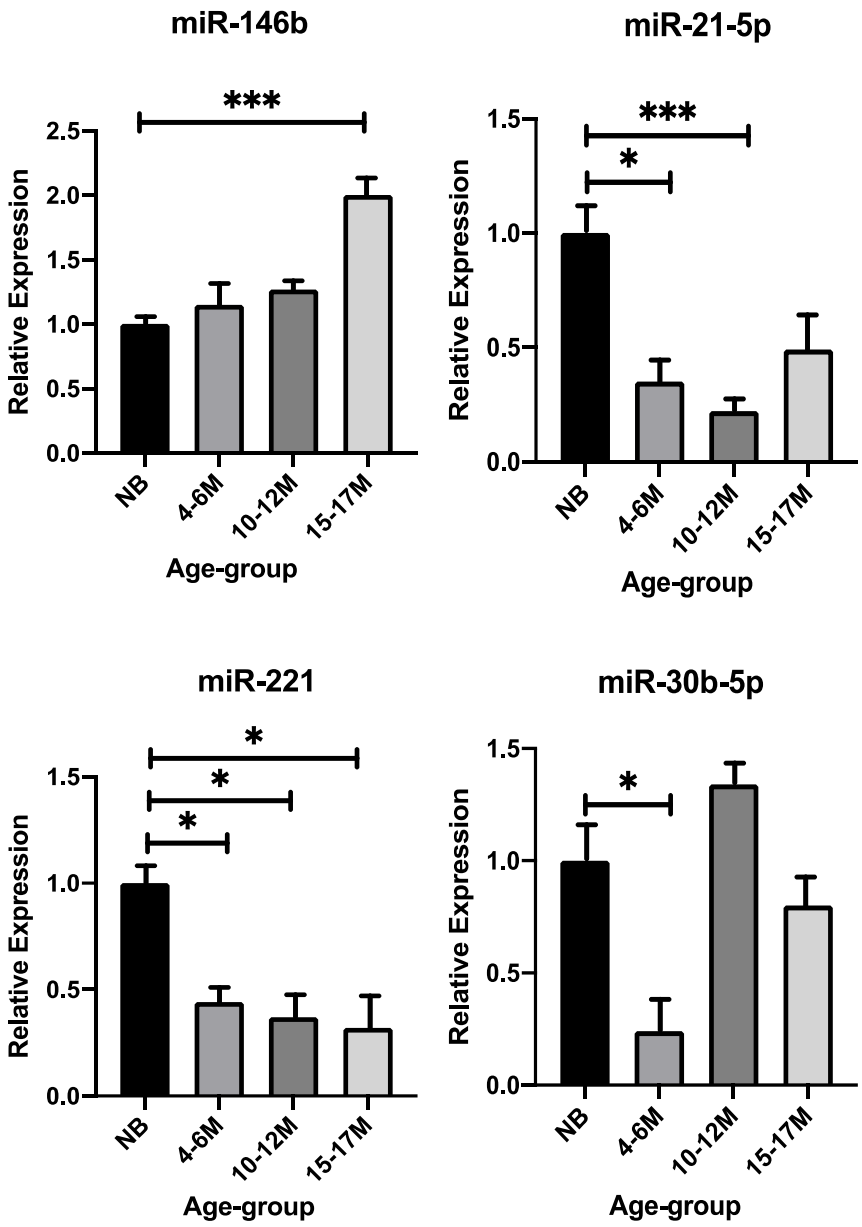


Figure 3.6 (a, b, c): qRT-PCR validation of DE-miRNAs in sequencing results. Plasma miRNAs have been shown as DE in an upward direction (a), downward direction (b) and not DE (c). All expression levels were normalized to miR-378. Relative expression is shown as fold change (mean± SEM) and statistical significance as *p<0.05, **p<0.005, ***P<0.0005.

The relative abundance of all selected miRNAs was quantified using miR-378 (stable number of reads and Cq values among the four groups) for normalization. The selected miRNAs were detected in all the samples. When compared with NB, the effect of the growing age was statistically significant for miR-10b, miR-143, miR-126-5p, miR-223 (Figure. 3.5-a, b) while for miR-99a-5p and miR-30a-5p no significant variations in expression were identified (Figure 3.6-c). MiR-10b, miR-143, and miR-126-5p showed an increase in the expression during growth and reached the peak at 4-6 and 10-12 months of age (Figure 3.6-a); whereas the expression of miR-223 remained significantly downregulated during the three growth periods (Figure 3.6-b).

a.

b.


miR-146b
***
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
NB
4-6M
10-12M
15-17M
Age-group
miR-21-5p
***
*
Relative Expression
1.5
1.0
0.5
0.0
NB
4-6M
10-12M
15-17M
Age-group
miR-221
*
*
*
Relative Expression
1.5
1.0
0.5
0.0
NB
4-6M
10-12M
15-17M
Age-group
miR-30b-5p
*
Relative Expression
1.5
1.0
0.5
0.0
NB
4-6M
10-12M
15-17M
Age-group

c.

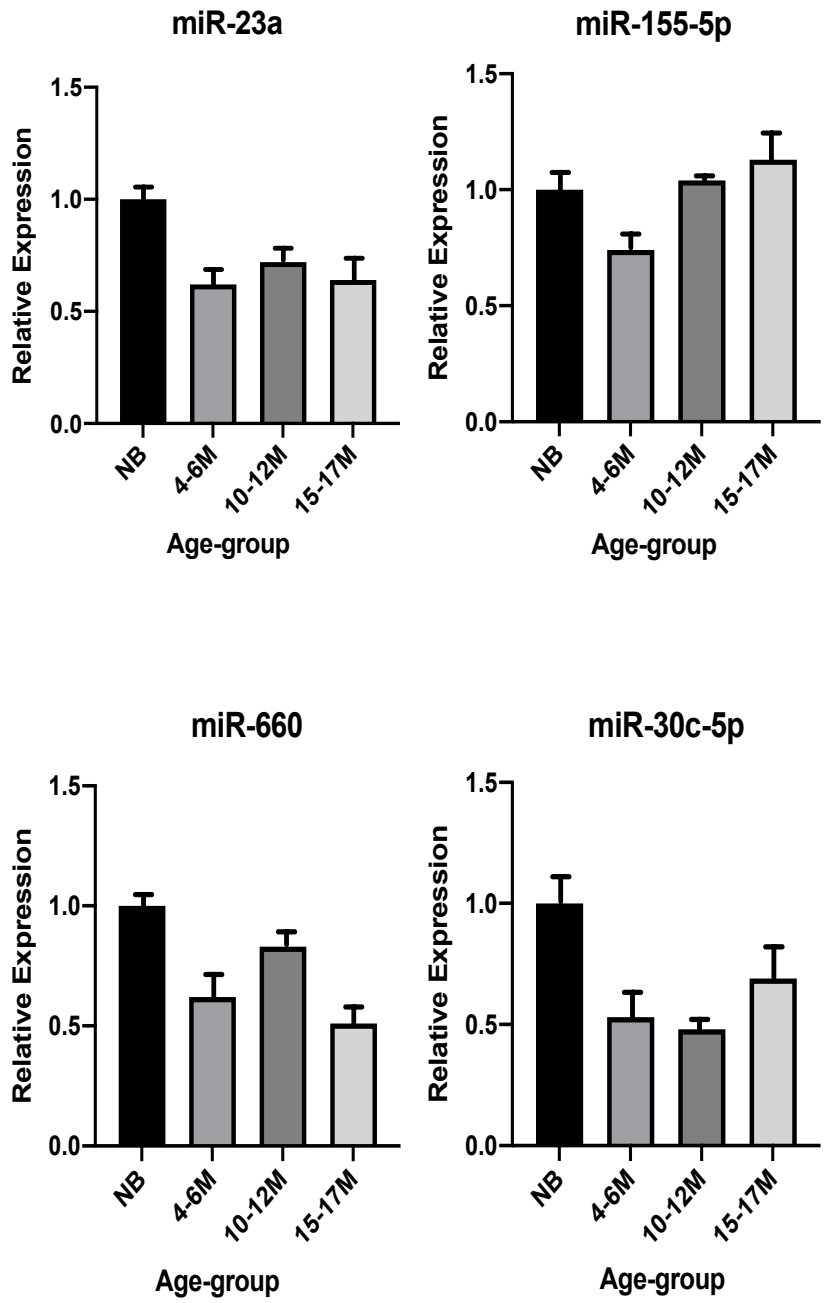


Figure 3.7 (a, b, c): qRT-PCR expression profiles of arbitrarily selected skeletal muscle-related miRNAs. Plasma miRNAs have been shown as DE in an upward direction (a), downward direction (b) and not DE (c). Relative expression is expressed as fold change (mean± SEM) and statistical significance as *p<0.05, **p<0.005, ***p<0.0005

Expression assay analysis of 8 arbitrarily selected miRNAs was performed, and qRT-PCR revealed that miR-146b-5p expression increased in 15-17M old animals when compared with NB (Figure 3.7-a), whereas the expression levels of miR-21-5p, miR-221, miR-30b-5p decreased with the animal's growth (Figure 3.7-b). MiR-23a, miR-155-5p, miR-660, and miR-30c-5p showed no significant differential expression among the groups (Figure 3.7-c).

3.3 Discussion

The first goal of this study was to characterize the plasma skeletal muscle-related miRNA signatures of Piedmontese beef cattle from the first week of life until the time of the commercial end of the fattening period before slaughter to integrate information between ci-miRNAs expression, muscle physiological age-growth, and animals' weight.

In my experiments, the level of myomiRNAs including miR-1, miR-133a/b, and miR-206 were found negligible in all the age groups on sequencing. Muroya and colleagues reported a similar result obtained by microarray analysis done in the plasma of 22 months aged Japanese beef cattle (Muroya et al., 2015). In literature, circulating levels of myomiRNAs are described as increased in patients affected by muscular dystrophy (Marozzo et al., 2020) and of rhabdomyosarcoma (Tombolan et al., 2020). The higher abundance in bloodstream of patients with skeletal muscle pathologies suggests a possible relation with the injury of the fibers. To avoid possible bias due to muscle injuries, my experimental trial was executed on animals housed in the same animal facility under better welfare conditions related to the building structures (fences, drinkers, feeder bunks length etc.), bedding material, animals' space availability, social stability of animals' group, and management and monitoring by the farm manager and trained stockmen.

Among the DE-miRNAs identified by qRT-PCR, miR-10b, miR-126-5p, miR-143, and miR-146b were found to be increased in expression (Figure 3.5-a) along the age-groups when compared with NB and reached the highest expression at 10-12 months for the first three miRNAs, and at 15-17 months of life for miR-146b (Figure 3.6-a).

Previously, miR-10b, miR-143 and miR-146b were described as involved in PI3K/AKT pathway in the skeletal muscle tissue and myoblast cells (Ge et al., 2019; Huang et al., 2019; Zhang et al., 2017). It seems that all these miRNAs negatively regulated the myoblasts proliferation. A

study reported that miR-10b significantly suppressed PIK3CA expression and decreased PI3K/Akt/mTOR pathway activity, promoting expression of TGF-β (Dai et al., 2018). Furthermore, miR-10b expression steadily decreased during myoblasts proliferation, but significantly increased during myoblasts differentiation in C2C12 mouse cell line by binding with NFAT5 gene (isoform of Nuclear Factor of Activated T cells) which is recognized as a key regulator in myoblast migration and differentiation (O'Connor et al., 2007).

Additionally, miR-10b was previously identified upregulated in grazing cattle plasma compared with grain-fed animals coincidently with the expression change of miR-10b in the *Longissimus dorsi.* This observation was correlated with possible alterations of skeletal muscle regulation through PTEN targeting (the phosphatase that converts phosphatidylinositol 3,4,5-triphosphat), involved in muscle cell differentiation and hypertrophy (Muroya et al., 2016).

Likewise, miR-143 was demonstrated to have a role in skeletal muscle and adipose tissues through the regulation of target genes involved in PI3K, IGF and FOXO signaling pathways such as IGF-I, IGF-II, IGF-IR, and IGFBPs (Ahmad et al., 2020; Blumensatt et al., 2014a; Hribal et al., 2003; Zhang et al., 2017). The pathway analysis prediction (KEGG) of my sequencing data revealed a relation between the same pathways and ci-miRNAs identified in bovine plasma (Figure 3.4).

In *in vitro* bovine cell models, miR-143 exerted important function in bovine myogenesis by targeting IGFBP5 (Insulin like growth factor binding protein 5) (Zhang et al., 2017), which is an important component of IGF signalling pathway (Lee et al., 2013), but also its expression increased in differentiating bovine satellite cells (Wang et al., 2015). At the end of pigs' fattening period, miR-143 expression was found upregulated in the psoas muscle, assuming its involvement in processes for the transformation of muscle fiber type (Zuo et al., 2015), but an important positive role of miR-143 was already identified in adipogenesis mechanisms (Chen et al., 2014) and recently demonstrated highly expressed in the adipose tissue of cows (Vailati-Riboni et al., 2017).

As per my findings, the increased levels of expression of circulating miR-10b and miR-143 during 10-12 months of age could be suggestive of their involvement in differentiation processes when the animal substantially gains the body weight (table-5.2) but the increase in miR-143 expression could be also linked with adipocyte differentiation. So, it can be

hypothesized that this trend might mediate the maintenance of the proliferating status of myoblasts during the first months of life and then induces the differentiation of muscle and adipose tissue at the end of the commercial fattening period. Indeed, according to Bonnet and colleagues, in cattle, the increase in the number and differentiation of adipocytes at the intramuscular level is described beginning from 12 months of age (Bonnet et al., 2010).

In addition, I found miR-146b to be differentially expressed in the plasma when NB subjects and the elder groups were compared (Figure 3.5-a) and it was positively correlated with the body weight gain during the four growth points (Figure 4.2). In mouse and chicken models, miR-146b was described as a positive regulator of myogenic differentiation, acting through multiple targets (e.g., Smad4, Notch1, and AKT1) (Khanna et al., 2014) but this function was demonstrated in tissue. At present, no information is available about the role of plasma miR-143 and miR-146b in cattle, but their differential expression among ages in the bloodstream might attribute to an important role in the regulation of growth mechanisms probably related also to skeletal muscle (Huang et al., 2019).

4. Aim 2: Investigate functional effect of selected ci-miRNAs on the target genes of interest

4.1 Pathways and genes linked with skeletal muscle hypertrophy

4.1.1 Major pathways controlling hypertrophic phenotype: The regulation of muscle mass and fiber size primarily reflects protein throughput, i.e., the balance between protein synthesis and degradation within the muscle fibers (Sandri et al., 2008; Yoshida et al., 2020). This balance is co-ordinately regulated by several pathways. For instance, two major branches of AKT signalling pathways: the AKT /mTOR pathway controls protein synthesis and the AKT/FOXO pathway controls protein degradation. mTOR is a downstream target of Akt and mTOR activity is highly correlated with the anabolic/catabolic balance (Rommel et al., 2001). Moreover, skeletal muscle hypertrophy is mediated by a central extracellular factor known as IGF-1 which binds to its receptor IGF1R to propagate signalling pathways via PI3-K/Akt/mTOR. IGF1–PI3K–Akt/PKB–mTOR pathway acts as a positive regulator of muscle growth and the myostatin–Smad3 pathway acts as a negative regulator. For example, muscle-specific inactivation of the IGF1 receptor impairs muscle growth due to reduced muscle fiber

number and size. Conversely, muscle-specific overexpression of IGF-1 causes muscle hypertrophy (Hocquette et al., 2010; Rommel et al., 2001). IGF-I and II are both involved in muscle cell differentiation. IGF-II exerts its effect by binding to the IGFI-R. Moreover, it has been advocated through past studies that IGF-1 also mediates hypertrophy through calcineurin (PPP3CA)/nuclear factor of activated T-cells (NFAT) signaling pathway (Semsarian et al., 1999). Additionally, studies suggested that MEF2C regulates the hypertrophic processes (Oh et al., 2005). A distinct substrate of Akt known as glycogen synthase kinase 3 beta (GSK3b) has been shown to be modulated during hypertrophy via the inhibition of its activity through Akt phosphorylation, and this inhibition may induce hypertrophy by stimulating protein synthesis independent of the mTOR pathway (Ribas et al., 2011). TEAD1 (transcription enhancer factor 1) which is a member of the TEA domain family and is constitutively expressed in cardiac and skeletal muscles in pigs, mice and humans regulates the expression of many skeletal muscle-specific genes in coordination with several co-factors including MEF2 (Yoshida et al., 2020).

4.1.2 Myostatin (MSTN)

MSTN belongs to the TGF-β superfamily and was initially called as growth and differentiation factor 8 (GDF-8). MSTN has a great impact on the growth and development of skeletal muscle, such that genetic deletions or mutations in the MSTN gene cause a remarkable increase in skeletal muscle mass (Lee et al., 2001). The muscle hypertrophy phenotype in the absence of MSTN is either naturally present in farm or domestic animals (gene mutation occurred) or has been induced in rodents through the silencing of the gene (Knockout: MSTN - null mice). Supporting evidence for the role that MSTN plays during myogenesis has been observed in several other studies that have assessed myostatin function during muscle growth and repair (Aiello et al., 2018; Qian et al., 2015). Functionally, MSTN regulates the activity of the Akt pathway negatively and promote the activity of ubiquitin–proteasome system to induce atrophy. In the case of Piemontese cattle, the gene is naturally mutated resulting in the unrestricted muscle development known as double-muscling. In this bovine breed, natural mutation in myostatin gene occurs whit the changing of guanine in adenine at position 938 (G938A), resulting in the substitution of a critical cysteine with tyrosine in the main signalling area of myostatin. The MSTN gene has been highly conserved throughout evolution and

comprises three exons and two introns. The Piedmontese MSTN sequence contains a missense mutation in exon 3 where tyrosine is substituted by an invariant cysteine in the mature region of the protein (R et al., 1997). At present, in Piedmont region it is estimated that 96% of animals of this breed carry the MSTN mutation in homozygous form (Coalvi - Consorzio Di Tutela Della Razza Piemontese - Fassone).

4.1.3 Insulin like Growth factor (IGF-1):

IGF-1 is one of the best-characterized growth factors, and its role was investigated in skeletal muscle tissue. IGF-1 has been shown to modulate muscle size and it is recognized as regulator of multiple muscle functions. IGF-1 can orchestrate both protein synthesis and degradation pathways, and changes in IGF-1 signaling in skeletal muscle can greatly affect myofiber size and function (Lu et al., 2017; Peters et al., 2017)

One of the most important functions of IGF-1 is its regulation of protein synthesis in skeletal muscle and promotion of body growth. Upon binding to IGF-1, IGF-1R phosphorylates an intracellular adaptor protein insulin receptor substrate-1 (IRS-1), which recruits and phosphorylates PI3K followed by Akt phosphorylation. The PI3K/Akt pathway plays a critical role in myotube hypertrophy (Yoshida et al., 2020)

4.2 Material and methods

4.2.1 Cell Culture

Primary bovine satellite cells/myoblasts (BoSC) were isolated and cultured from bovine *longissimus dorsi* muscle, as previously reported (Miretti et al., 2017) and 293T cell line (ATCC, Rockville, MD, USA) was obtained from the American Type Culture Collection. 293 T cells and myoblasts were cultured in growth medium (GM) containing high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, USA), supplemented with 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, USA) and 10% fetal bovine serum (FBS) (Euroclone, Pero, MI) and 20% FBS plus 10% Horse Serum (Sigma-Aldrich, St. Louis, USA) respectively. Cells were incubated at 37°C in a 5% CO2 humidified atmosphere. The myoblasts differentiation was induced by replacing GM with differentiation medium (DM) (DMEM 2% horse serum) when myoblasts confluence reached nearly 70–80%.

4.2.2 Transfections

293 T cell line and myoblasts were used to perform transfections for dual-luciferase assay. Cells were seeded at the count of 100000 cells per well of a 24-well plate. At 60-70% confluence cells were transfected. A total of 300 ng of the appropriate psicheck-2 luciferase reporter construct cloned with 3'-UTR of the MSTN transcript (Miretti et al., 2013) was co-transfected with 30 nM final concentration of double-stranded RNA oligonucleotides designed to mimic miR-23a, miR-126-5p (Qiagen, Hilden, Germany) and miR-27b (Exiqon, Woburn, USA) molecules using Lipofectamine 2000 (Invitrogen, Waltham, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, the medium was changed, and cells were grown for additional 24h before assay. Psicheck-2 vector was commercially obtained from Promega (USA).

To study the effect of selected miRNAs on their target gene expression**,** myoblasts were cultured in 6-well plates in GM and were transiently transfected with 30nM of specific mimics and 100nM of inhibitors of miR-23a and miR-143 (Qiagen, Hilden, Germany) along with a negative control scramble (Exiqon, Woburn, USA) using Lipofectamine 2000 (Invitrogen, Waltham, USA). After 24h, BoSC were harvested for the expression analysis of MSTN and IGF1R by qRT-PCR.

Table 4.1: miRNA mimics and inhibitors used for transfection of myoblasts

miRNA	**mimic**	**inhibitor**
miR-126-5p	MIMAT0000444: 5'CAUUAUUACUUUUGGUACGCGssss	MIMAT0000444: 5'CAUUAUUACUUUUGGUACGCG
miR-23a	MIMAT0000078: 5'AUCACAUUGCCAGGGAUUUCC	MIMAT0000078: 5'AUCACAUUGCCAGGGAUUUCC
miR-143	MIMAT0000435: 5'UGAGAUGAAGCACUGUAGCUC	MIMAT0000435: 5'UGAGAUGAAGCACUGUAGCUC

4.2.3 Luciferase Assay

Luciferase assay Firefly and Renilla luminescent signals arising from psicheck-2 transfected cells were quantified according to the manufacturer's instructions using Dual-Luciferase

Reporter Assay System (Promega, Madison, USA) with a VICTOR Multilabel Counter luminometer (PerkinElmer, Waltham, USA). All values are given relative to transfections with the appropriate negative (Scramble) and positive (miR-27b) controls. The psiCHECK™-2 Vector was designed to provide quantitative and rapid optimization of RNA interference (RNAi) and enabled monitoring of changes in expression of the target gene MSTN. In the vector, Renilla luciferase was used as the primary reporter gene, and the gene of interest was cloned into a multiple cloning region located downstream of the Renilla translational stop codon. Initiation of the RNAi process by synthetic miRNA mimic towards a gene of interest resulted in cleavage and subsequent degradation of the bound mRNA. Measuring decrease in Renilla activity provided a convenient way of monitoring the RNAi effect.

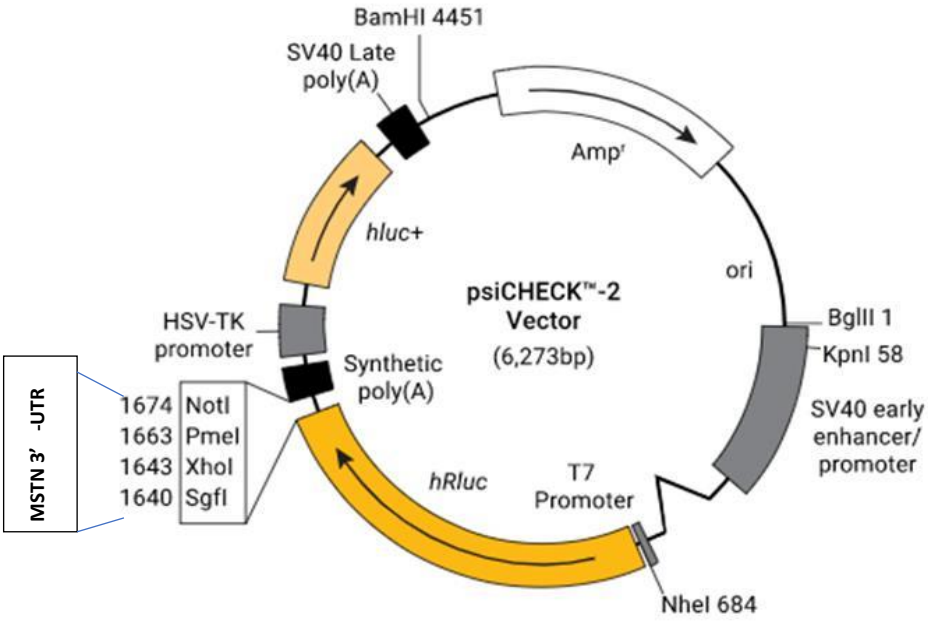


Figure 4.1 psiCHECK™-2 Vector with luciferase reporter construct cloned with 3'-UTR of the MSTN transcript

DNA constructs: The 3'-UTR of the MSTN transcript was amplified from genomic Piedmontese bovine DNA by PCR using forward 5'- GGTCTATATTTGGTTCATAGCTTCC -3' and reverse 5'- TCTTTCAAAAAAGGTGAAAACAC -3' primers, cloned into the Cloning vector pJET (Clone Jet PCR Cloning kit, Fermentas), and sequence was confirmed. The predicted bovine miR-27b seed-site was mutated with mutagenic primers by using the Quick-Change Multisite

mutagenesis kit (Stratagene, Waldbronn, Germany); mutations were confirmed by sequence analysis. The wild-type and mutated 3'-UTRs were subcloned into the NotI–XhoI site of the psicheck-2 vector (Promega, Madison, USA) (Miretti et al., 2013). Psi-check2 reporter vector containing wild type or mutant MSTN 3'-UTR contains Renilla luciferase reporter gene, hRluc, which is used to monitor changes in expression of 3'-UTR as the result of miRNA binding. The firefly luciferase reporter gene, hluc, is used to correct luciferase signal for transfection efficiency. The 1500-bp MSTN 3'-UTR sequence containing miR-27b consensus sequence (wild type or mutated) was inserted into the vector at the 3' end of the hRluc reporter gene.

4.2.4 RNA extraction and cDNA synthesis

For the RNA extraction from myoblasts, TRI Reagent (Sigma-Aldrich, St. Louis, USA) was used following the manufacturer's protocol. Cells were homogenized in 1ml of TRI reagent and RNA pellets were resuspended in variable amount of RNAse free water corresponding to the size of pellet. RNA quantity and quality were determined using Nanodrop ND1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The ratio of the optical densities measured at 260 and 280 nm was > 1.9 for all the RNA samples. cDNA was synthesized using miScript Reverse Transcription Supermix (Qiagen, Hilden, Germany) for qRT-PCR kit (Bio-Rad, Hercules, USA) for each sample taking the volume of eluted miRNAs sample equivalent to 500ng.

4.2.5 Quantitative assessment of miRNAs and mRNA target gene expression

qRT-PCR with total cDNA was performed using SYBR green II PCR Kit (Qiagen, Hilden, Germany). PCR amplifications were performed on Bio-Rad CFX Connect Real-Time System (Bio-Rad, Hercules, USA). The qRT-PCR parameters specific for miRNAs and gene expression are detailed in Table 3. Differential expression among miRNAs was carried out by comparing the normalized Cq values (ΔCq) for all biological replicates between the two group of samples. Based on stable number of sequencing reads and stable value of qRT-PCR expression among the groups, miR-378 was selected as the reference gene. The fold change of expression of gene/miRNA was calculated by $2^{-\Delta\Delta Cq}$ method where ΔCq = Cq of the target gene/miRNA−Cq of the reference gene/miRNA (Miretti et al., 2017). Data were expressed as fold-change with respect to NB samples.

For the quantitative expression analysis of target genes, cDNA (5 µg) was prepared in a single run to perform qRT-PCR experiments for all the selected genes. To determine the relative amount of specific IGF1R and MSTN transcripts (Table 2), the primers for target and reference genes were designed on Bos taurus GenBank mRNA sequences using Primer 3 Software (version 4.0). Oligonucleotides were designed to cross the exon/exon boundaries to minimize the amplification of contaminant genomic DNA and were analysed with the IDT tool (SciTools Web Tools, n.d.) for hairpin structure and dimers formation. Primer specificity was verified with BLAST analysis against the genomic NCBI database. To establish primers efficiency, the dilution method was used. Hypoxanthine phosphoribosyl transferase 1 (HPRT-1) gene was used as a reference gene for RNA concentration and reverse transcription efficiency (Miretti et al., 2017). Table 4.1 gives information about miRNA mimics and miRNA inbitors (miRCURY SALNA, Qiagen, Maryland, USA) used for transfection and table 4.2 summarizes primer assays information including sequences and target gene assays with gene accession number and amplicon sizes. Primers were pre-designed and commercially synthesized by LNA technology (Qiagen, Maryland, USA)

Table 4.2: Primer assays for target genes

Gene	Accession No.	5' -3' sequence	Amplicon size
IGF1R	NM_001244612	F: GGACGCAGTACGCCGTTTAC R: AGGGAGGGCGGGTTCCACTT	187
MSTN	NM_001001525	F: GGACGCAGTACGCCGTTTAC R: AGGGAGGGCGGGTTCCACTT	256
HPRT1	NM_0001942	F: CGAGATGTGATGAAGGAGATGG R: TGATGTAATCCAGCAGGTCAGC	132

Table 4.3: qRT-PCR parameters for miRNA assay and mRNA expression analysis

Target to be quantified	qRT-PCR parameters	Kit used
Target gene expression	1. 94°C for 30 sec 2. 95°C for 10 sec 3. 60°C for 30 sec 4. 75°C for 5 sec 5. 95°C for 5 sec Repeat steps 2, 3 and 4 for 39 cycles	Bio-Rad (Hercules, CA, USA)

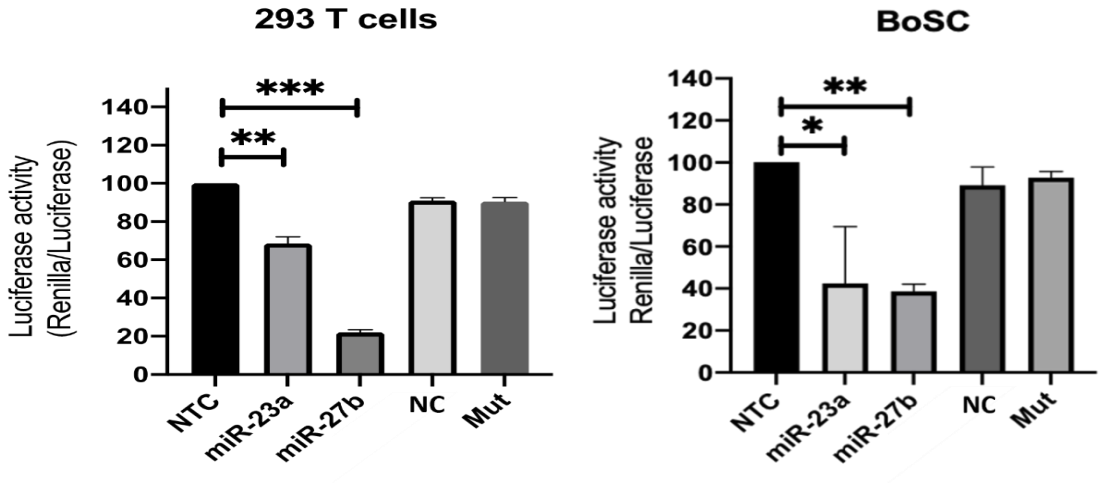
miRNA and gene expression in satellite cells	1. 95°C for 15 min 2. 94°C for 15 sec, slow ramp rate to 1.0 C/sec 3. 55°C for 30 sec, slow ramp rate to 1.0 C/sec 4. 70°C for 30 sec, slow ramp rate to 1.0 C/sec Repeat all the steps for 40 cycles	SYBR Green PCR Kit (Qiagen, Hilden, Germany) compatible with cDNA prepared with miSCRIPT II RT kit (Qiagen, Hilden, Germany)

4.3 Results

4.3.1 miR-23a and miR-126-5p directly target the 3'-UTR of bovine MSTN

Among 14 selected ci-miRNAs, TargetScan version 7.2 predicted MSTN as one of the target genes for miR-23a and miR-126-5p. To investigate the mechanism of miR-23a and miR-126-5p, transfection of miRNAs mimic with luciferase reporter gene cloned to the wild-type 3'-UTR of MSTN and mutated MSTN 3'-UTR were performed in both 293T cells and BoSC. miR-27b mimic and scramble were used as positive (Miretti et al., 2013) and negative controls, respectively. MiR-23a significantly inhibited the dual luciferase activity (34%, $p < 0.05$ and 57%, $p< 0.05$ of wild-type MSTN 3'-UTR, but not of MSTN 3'-UTR-mutated confirming the binding between miR-23a and MSTN in 293 T cells and BoSC systems, respectively. MiR-126-5p significantly inhibited the activity by 11% in 293 T cells and by 43% in BoSC indicating that MSTN is the direct target of miR-126-5p.

a.



b.

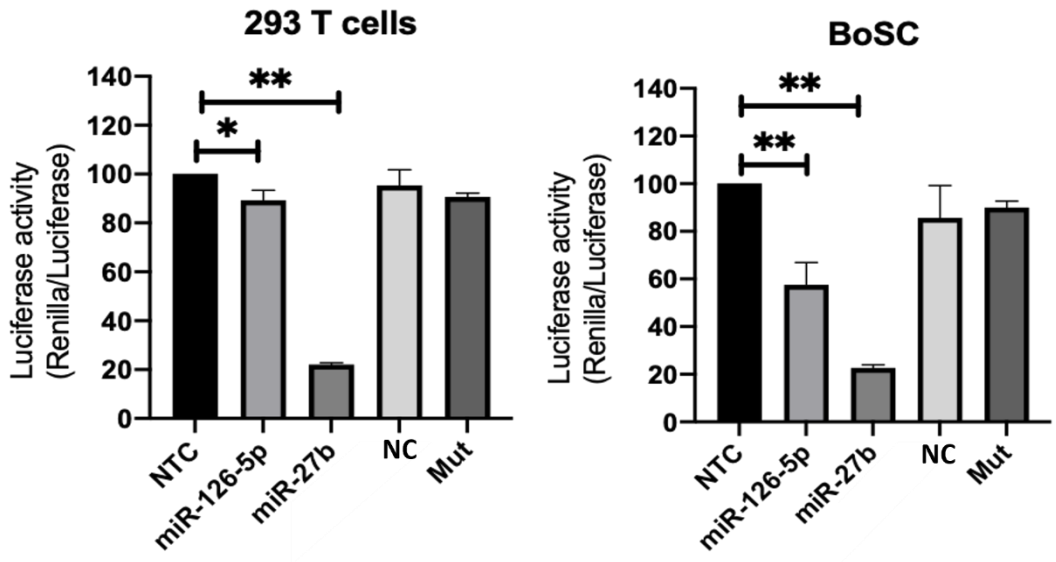


Figure 4.2: Luciferase assay to confirm MSTN as a true target of miR-23a and miR-126-5p. 293 T and BoSC cells were co-transfected with psi-check 2 reporter vectors containing native or mutated MSTN 3'UTR and miR-23a and miR-126-5p mimics followed by cell lysis and luciferase assay 24h later. Statistical significance was calculated for p<0.05. (NTC: Non-treated cells; Mut: MSTN 3'-UTR mutated). Scramble was used as negative control (NC). a, b Dual luciferase activity inhibition by miR-23a in 293 T cells (34%, p < 0.05) and BoSC (57%, p < 0.05). c, d Dual luciferase activity inhibition by miR-126-5p in 293 T cells (11%, p<0.05) and BoS C (43%, p<0.05).

4.3.2 miR-23a and miR-143 overexpression modulates skeletal muscle hypertrophy-related target genes

To test the ability of miR-23a and miR-143 to alter the expression of their targets in satellite cells under GM conditions, the overexpression of both miRNA mimics was induced and the post-transfection expression of miR-23a and miR-143 and their target genes MSTN and IGF1R respectively, at mRNA levels were defined through qRT-PCR.

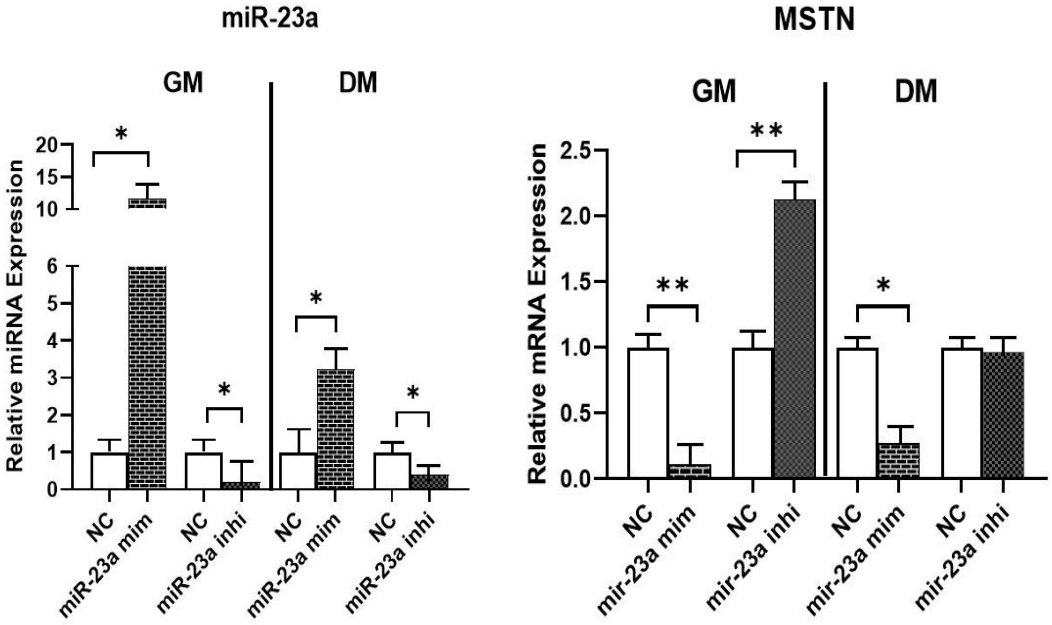


Figure 4.3 (a, b) Expression of miR-23a and target gene MSTN screened by qRT-PCR. Upregulation or downregulation of miR-23a levels affected the expression of MSTN gene expression in BoSC when transfected with miR-23a mimic, miR-23a inhibitor and compared with scramble (NC). Differences between the expression level of negative control and miR-23a mimic and inhibitor were assessed by student t-test. Differences in gene expression are shown as fold change (mean ± SEM). Significance expressed as *p<0.05, **p<0.005

The results showed that the overexpression of miR-23a (log2fold change = 11, $p < 0.05$) induced a significant decrease in MSTN expression ($p<0.004$, Figure 4.3-a) in proliferative BoSC. Conversely, knockdown of miR-23a through miR-23a inhibitor (log2fold change = 0.02, $p < 0.05$) increased MSTN expression ($p<0.002$; Figure 4.3-b). These results revealed that the overexpression of miR-23a inhibited MSTN mRNA expression in BoSC.

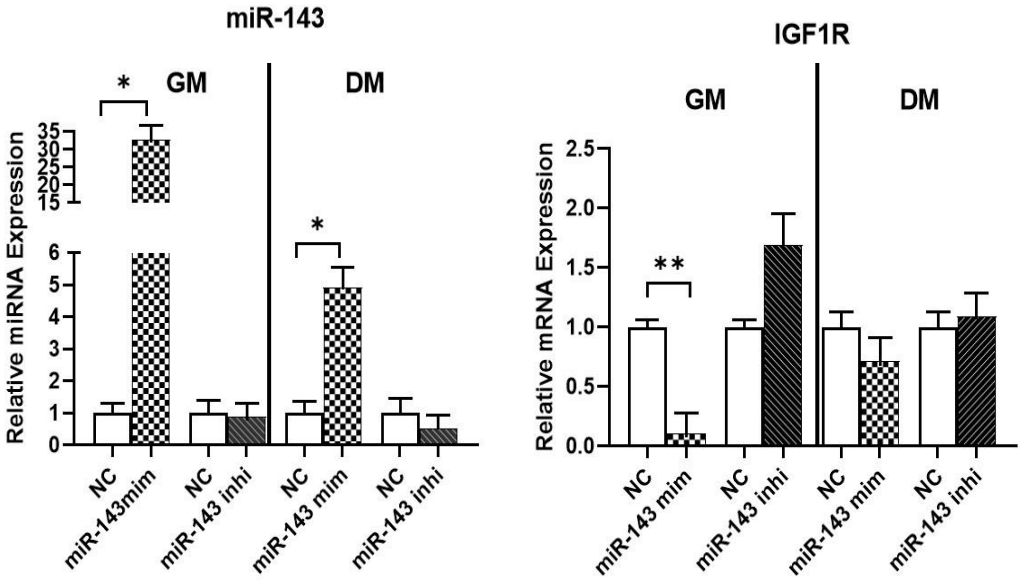


Figure 4.4 (a, b) Expression of miR-143 and target gene IGF1R measured by qRT-PCR. Upregulation or downregulation of miR-143 levels affected the expression of IGF1R gene expression in BoSC when transfected with miR-143 mimic, miR-143 inhibitor and compared with scramble (NC: negative control). Differences between the expression level of negative control and miR-143 mimic and inhibitor were assessed by student t-test. Differences in gene expression are shown as fold change (mean ± SEM). Significance expressed as *p< 0.05, **p< 0.0005.

Comparable results were obtained with the overexpression and the inhibition of miR-143 with significant different levels of IGF1R expression when compared with control after 24 h of transfection (Figure 4.4-a, b). Overexpression of miR-143 (log2fold change = 32, $p < 0.05$) significantly reduced the expression of IGF1R, whereas inhibition of miR-143 (log2fold = 1.89, $p < 0.05$) showed higher expression of IGF1R.

4.4 DISCUSSION

MSTN is a secreted growth and differentiation factor that belongs to TGF-β superfamily. MSTN gene mutation or targeted degradation of its mRNA has shown increased muscle mass attributing to muscle hypertrophy phenotype (Miretti et al., 2013). Several published results suggested that MSTN inhibits myoblast proliferation (Liu et al., 2020; Taylor et al., 2001; Thomas et al., 2000). On the other hand, literature defined IGF-I and IGF-II and their receptor

as crucial mediators of skeletal muscle development, hypertrophy, and regeneration (Galvin et al., 2003; Meyer et al., 2015; Quinn et al., 2007).

In qRT-PCR assay, miR-23a showed high expression in terms of Cq values in all the age-groups. Even if not differentially expressed, this result combined with the targeting of MSTN mRNA by miR-23a is suggestive of an important role of this miRNA in Piedmontese breed affecting the double-muscled phenotype. A previous study has shown the functional effect of miR-27b on MSTN and as one of the possible contributing factors of hypertrophy in Piedmontese cattle (Miretti et al., 2013). Here I have demonstrated for the first time the direct binding between miR-23a and MSTN gene. Indeed, both in cardiac (K. Wang et al., 2012) and in skeletal muscle (Wada et al., 2011) it was previously shown that miR-23a can mediate the hypertrophic signals, through the binding of an anti-hypertrophic protein (NFATc3) and two muscle-specific ubiquitin ligases (MAFbx/atrogin 1 and MuRF1) that promote hypertrophy by protecting muscle atrophy-associated protein degradation. These findings suggest that the high level of miR-23a expression in Piedmontese breed may have a role in the modulation of skeletal muscle mass. Future studies are required to specifically elucidate the role of miR-23a and if it can exert a synergic effect with other miRNAs such as miR-126-5p and miR-27b in the hypertrophic program.

Focusing on miR-143, in the current study, IGF1R is found as the putative target gene of miR-143. The results revealed that upregulation of miR-143 downregulates IGF1R expression mainly in growth medium condition when myoblasts tend to proliferate more instead of differentiating. According to Targetscan database miR-143 targets a bunch of putative genes, which include IGF-I, IGF-II, IGF-IR, and IGFBPs, that are involved in the IGF signaling pathway (Zhang et al., 2017). In a study, IGFBP5 was validated as a target gene of miR-143 (Cordes et al., 2009). The results revealed that upregulation of miR-143 downregulates IGFBP5 expression, arresting myoblasts differentiation, thereby resulting into a fewer myotubes when compared with control. In contrary, inhibition of miR-143 upregulates IGFBP5 expression, promoting myoblasts differentiation, resulting in an increased number of myotubes. Another study showed that miR-143 and miR-145 perform in coordination to promote differentiation while restraining the proliferation of smooth muscle cells (Cordes et al., 2009). miR-143 was also reported to negatively regulate myoblast development in human

and mouse myoblasts in an age-related muscle regeneration study (Soriano-Arroquia et al., 2016).

5. Aim 3: Relationships between miRNAs expression and animals' body weight

To further deepen the relationship between the animal weight and the expression of miRNAs, we analysed the Spearman's rank correlation coefficients. Considering all the age points, the statistical test highlighted that miR-126-5p and miR-146b were positively correlated to animals' weight, rho = 0.26 (p = 0.02) and rho = 0.36 (p = 0.002) respectively, while a negative correlation was observed for miR-223 expression and beef cattle weights, rho = -0.39 (p = 0.001) (Figure 5.1, 5.2, 5.3). The correlation between body weight and expression of miR-126-5p and miR-146b was positive (Figure 5.1, 5.2), whereas the correlation was negative for miR-223 (Fig 11).

Table 5.1 Spearman correlation coefficient values and the pValue for the expression of 14 selected miRNAs and animal weight (kg) and the expression of 14 selected miRNAs

miRNA	Spearman	pValue
miR-223	**-0.392168**	**0.00103**
miR-146b-5p	**0.3679788**	**0.002187**
miR-126-5p	**0.266183**	**0.02946**
miR-10b-5p	0.2219483	0.07106
miR-660	-0.23236	0.05847
miR-30a-5p	0.2345063	0.05612
miR-143	0.2177467	0.07671
miR-221	-0.181477	0.1416
miR-21-5p	-0.145908	0.2387
miR-30b-5p	0.119285	0.3363
miR-23a	-0.123595	0.319
miR-99a-5p	-0.092206	0.458
miR-30c-5p	0.0808416	0.5155
miR-155-5p	0.0374455	0.7635

Table 5.1 shows the values of Spearman correlation coefficient established between the body weight (kg) of the animals during four age points and expression (2^-ΔCq) of selected 14 miRNAs for validation. Body weight of the animals in Kgs (table 5.2) was recorded in the data base of animal facility, Dept. of Veterinary Science, Turin, Italy.

Table-5.2 Body weight (Kg) of the target animals recorded during four age points

NB	4-6M	10-12M	15-17M
36	237	422	495
39	230	333	426
40	202	330	430
33	192	372	536
34	265	380	437
34	224	430	595
36	197	325	408
37	302	340	507
35	285	323	466
34	188	342	448
40	195	371	521
37	261	481	602
34	247	340	430
34	234	352	445
38	245	408	635
42	228	380	450
47	231	351	412
41	252	420	509
48	212	388	515
30	205	392	467
43	287	416	603
38	275	401	552

Table 5.2 gives the detail of the body weight of the target animals including both the genders. The body weight of the animals in Kgs was recorded during different growth periods in the data base of animal facility of the department of veterinary science, University of Turin, Italy.

5.1 Discussion

Interestingly, a positive correlation between the expression of miR-126-5p and miR-146b and the increasing body weight of the animals could be suggestive of their role in the positive regulation of myogenic differentiation, acting through multiple targets (e.g., Smad4, Notch1, and AKT1) (Koutalianos et al., 2021) and as the animal ages, the body mass increases as a result of an increase in the size of muscle fibers attributed to more number of myoblasts getting differentiated. Referring to miR-126, a study provides evidence that miR-126 cause aberration in the transcriptional regulation in healthy old males compared with healthy young males thereby having an influence on the aging and muscle plasticity. The study shows that miR-126 in myocytes, in vitro, has direct effects on the expression of regulators of skeletal muscle growth and activation of insulin growth factor 1 (IGF-1) signaling (Rivas et al., 2014) indicating its role in the differentiation of myocytes rather than the proliferation. Zhou and group in 2020 detected the presence of miR-126 and miR-146 in healthy adults upon physical activity (Zhou et al., 2020). In my knowledge there is no information available about the expression variation of skeletal muscle related plasma miRNAs linked with age in livestock.

Whereas it could be more interesting to look deeper into the negative correlation between the expression of miR-223 and the body weight of the animals. A latest study stated that miR-223-3p plasma levels are elevated in myotonic dystrophic mice in comparison to healthy and could be one of the causative agents of myotonic dystrophy which is the most common muscular dystrophy in adults and is characterized by muscle wasting (Koutalianos et al., 2021). Furthermore, according to another study the lower levels of miR-223-3p in skeletal muscle tissues could affect the expression of its targets, thus causing downstream effects such as insulin resistance (Taïbi et al., 2014). Song et al cultured vascular smooth muscle cells under dynamic or static conditions to demonstrate that miR-223 is downregulated under stretch stress and confirmed that miR-223 levels reduce four-fold under stretch stress conditions compared to static conditions. Overexpression of miR-223 cause the inhibition of protein levels of IGF-1R. Hence, stretch stress induces vascular muscle cells proliferation through

increased IGF-1R expression and inhibit PI3K–Akt signaling (Song et al., 2012). Following results give the impression that these miRNAs may have an influence on the skeletal mass increase with the age growth, but further experiments are due to better understand the related mechanism.

On the other hand, remaining 11 selected miRNAs did not show significant correlation between the plasma expression and the body weight gain during four growth periods.

Conclusion

In the recent years, miRNAs and more specifically ci-miRNAs have gained a lot of attention due to their legit fine-tune role in orchestrating several physiological processes, metabolic changes and adaptive response pathways underlying skeletal muscle growth, differentiation, and hypertrophy (Butchart et al., 2016; Y. Dai, Zhang, et al., 2016; S. Lee et al., 2017c; Siracusa et al., 2018). However, mostly the studies have been carried out on tissue miRNAs in humans and rodent models (Bazgir et al., 2016; Horak et al., 2016). The knowledge about ci-miRNAs in livestock and particularly in bovine is limited. To characterize the changes in skeletal-muscle related ci-miRNAs during the growth of Piedmontese beef cattle, I examined the plasma samples of animals categorized into four groups based on age and corresponding weight.

For the first time, the findings of this study provide evidence of high numbers of skeletal-muscle related miRNAs in beef cattle plasma of different age groups. Among these ci-miRNAs, miR23a and miR-126-5p were demonstrated to directly bind MSTN mRNA in a bovine satellite cell *in vitro* model. Three DE ci-miRNAs (miR-146b, miR-126-5p and miR-223) have shown a correlation between miRNAs expression value and body weight during different ages. All these results are the basis for future investigations about the possible role of ci-miRNA as a biomarker of muscle post-natal growth and development. Remarkably, the information about plasma miRNAs associated with production traits in beef cattle could prove to be promising biomarkers for the genetic selection of meat-purpose animals for future breeding. The pattern of miRNAs expression and its correlation with age growth status are, obviously, intriguing to predict at calf birth and the trend of weight during the first months of life.

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