**PILOT STUDY ON CIRCULATING MICRORNAS PROFILE IN PIEDMONTESE CATTLE DURING DIFFERENT GROWTH PERIODS**

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# Pilot study on Circulating microRNAs profile in Piedmontese Cattle during different growth periods

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***Abstract***
MicroRNA (miRNA)are small (21-23nt) non-coding, highly conservative and tissue specific RNA that regulate geneexpression after transcription and their circulating levels can be measured and thus could be potentially used as biomarker for various physiological changes. However, the role of circulating miRNAs in livestock species has not been fully studied.Therefore, our pilot study aimed to explore and describe expression profile of miRNAs in plasma samples during different growth periods.Out of 24 RNA samples processed, we have used one RNA sample from each age group (newborn calf, 5 month/weaning, 11 month and 16 month of age) for sequencing.Using small RNA sequencing (NGS), we have identified total 1031 miRNAs of which369 miRNAs were expressed in all samples (n = 4 bovine).Among miRNAs identified in plasma, we have found a lot of miRNAs closely related to myoblast proliferation or myocyte differentiation such as miR-1, miR-206, miR-26a and miR-27b.These results provide novel information on bovine circulating miRNAs in Piedmontesebreed but this is pilot study and further analysis is needed.

***Key words:***cattle, circulating miRNA, skeletal muscle

## INTRODUCTION

Piedmontese cattle is a native-breed of North-West Italy and is characterized by a peculiar muscular hypertrophic development called double-muscle. Genesis of skeletal muscle is a complex process comprising of proliferation, differentiation, and formation of myotubes and myofibers. A set of postnatal muscle stem cells (satellite cells) produce myoblast cells that can fuse with the existing myofibers, thus increasing their size(Robelin et al, 1993). These molecular events are regulated by myogenic regulatory factors (e.g. MyoD) and microRNAs (miRNAs)(Bukingham et al, 2014). MiRNAs are small non-coding RNAs, 21-23 nt in length, that regulate gene expression and play key roles in various physiological and developmental processes(Bartel et al, 2004). miRNAs highly enriched in muscle are called myomiRNAs. The main known myomiRNAs are miR-1, miR-133a/b and miR-206. These miRNAs have important roles in regulating myogenesis and differentiation of skeletal muscle (Kim et al., 2006). Other miRNAs such as miR-24, miR-26a, miR27b, miR125b, miR148a, miR-181, and miR-214 have been reported to regulate myoblast physiology but are not particularly abundant in the muscle tissue (Liu et al., 2010). MiRNAs can be also released from the cell and circulate in the blood or other body fluids in a stable cell-free form (ci-miRNAs). Recent studies show that ci-miRNAs participate in cell-to-cell communication to regulate normal and pathological processes indicating that ci-miRNAs can be used as biomarkers of physiological or pathological processes(Stenfeldt et al, 2017; Turchinovich et al, 2012). In livestock, ci-miRNAs have not been totally explored. So, studying the role of ci-miRNAs associated with the skeletal muscle growth could be useful for developing strategies to improve production performance in livestock.
Previous studies indicated that circulating levels of tissue specific miRNAs could be measured and used as biomarker for tissue functions and production traits and also for the early diagnosis of animal diseases(Ioannidis, 2018; Farrell, 2015).Therefore, we hypothesize that

plasma miRNA expression profiles are affected by age and associated with production traits in beef cattle and that ci-miRNAs could be promising biomarkers for the genetic selection of meat-purpose animals for future breeding.
The aim of this pilot study was to explore and define a panel of expression of ci-miRNAs in the plasma samples collected from the Piedmontese breed cattle just after the birth and during the course of skeletal muscle growth until slaughter. This study is part of a larger project that aims to identify if molecular factors such as specific ci-miRNAs could be effective biomarkers associated with skeletal muscle growth and production traits.

## MATERIALS AND METHODS

### Animals and collection of samples

For our project, blood samples are to be collected from 72 animals falling into four age groups (new born calf, 5 month/weaning, 11 month and 16 month of age) with 18 animals in each group and so far samples have been collected from 24 animals (6 samples for each age group) in Plasma EDTA vacutainers. Sample size was calculated using the online software- Power and Sample Size Calculation-PS, Vanderbilt University.
Animals were housed in the animal facility of the Dept of Veterinary Science, University of Turin, under the same management conditions. All the experiments involving Piedmontese cattle were approved by the Ethical Animal Welfare Committee of the Dept. of Veterinary Science, UniTO (Prot. No. 663).

### MiRNA and total RNA extraction

Blood samples were processed within 2 hr of collection according to a standardized operating procedure (centrifugation at 3500 rpm for 10 min at 22°C) to collect plasma. MiRNA and total RNA were extracted with miRNeasy Plasma/serum kit (Qiagen, Germany) according to the manufacturer's protocol where Ce_miR39 was added as spike-in control. Quantification of miRNA was done using QuBit 3.0 fluorometer (Invitrogen, Life technologies, USA) and the samples with sufficient quantity of miRNA were immediately reverse transcribed into cDNA (Qiagen/Exiqon). The quality of RNA extraction and reverse transcription was checked with Real-time qPCR using Ce_miR39 primer assay.

### Library preparation and sequencing

TruSeqSmallRNA Sample Prep kit (Illumina, San Diego, CA) was used for library preparation following the manufacturer's instructions. Just before the library preparation, RNA samples were again quantified and quality tested by Agilent 2100 Bioanalyzer RNA (Agilenttechnologies, Santa Clara, CA) or by Caliper RNA LabChip GX (Caliper Life Sciences, Hopkinton, MA).
Libraries were then prepared for sequencing and sequenced on single-end 75 bp mode on NextSeq500 (Illumina, San Diego, CA). Final libraries were quantified using the 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, MA).

### Sequencing data analysis

The Bcl2Fastq 2.0.2 version of the Illumina pipeline was used to process raw data for both format conversion and de-multiplexing. Whole data was normalized using the standard method by DESeq2.

**RESULTS AND DISCUSSION**

Out of 24 RNA samples processed, we used one RNA sample from each age group for next generation sequencing (NGS).

The quality of sequencing reads was assessed by the read length (Fig 1)

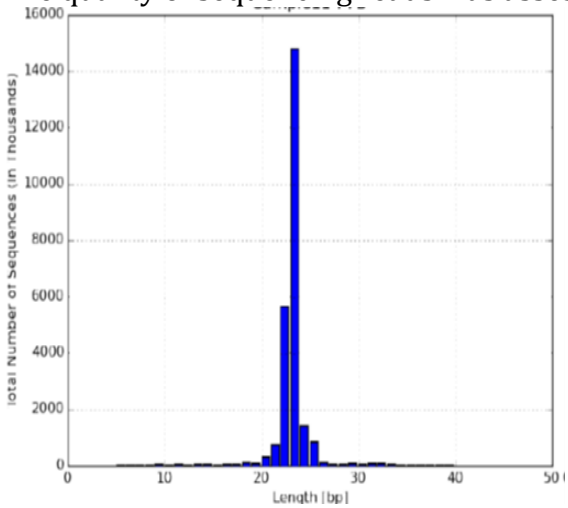


*Figure 1The length distribution of all sequence reads (example of one sample)*

Identified miRNAs matching sequence reads as determined with up to two mismatches allowed are as follows

| Age group | Known miRNAs | Known matched | Matched % |
|---|---|---|---|
| Newborn | 3424 | 1932 | 56.43 |
| 05 month | 3424 | 2327 | 67.97 |
| 11 month | 3424 | 895 | 26.14 |
| 16 month | 3424 | 1171 | 34.20 |

| **Age in months** | **0 month or newborn** | **05 month** | **11 month** | **16 month** |
|---|---|---|---|---|
| miR-1 (Koutsoulidou et al, 2011) | 32.14 | 10.02 | 35.3 | 14.5 |
| miR-206 (Kim et al, 2006) | 9.84 | 0.52 | 76.49 | 1.61 |
| miR-133a (Koutsoulidou et al, 2011) | 30.18 | 3.16 | 76.48 | 5.23 |
| miR-27b (Christ et al, 2009) | 85319.92 | 121582.8 | 72960.99 | 122924.14 |
| miR-221 | 7699.87 | 7435.64 | 6724.81 | 29248.14 |
| miR-181 b | 9322.38 | 7613.92 | 6654.21 | 5923.51 |
| miR-26a | 263241.72 | 333975.31 | 46997.20 | 582001.53 |
| miR-214 | 33.46 | 20.04 | 5.88 | 27.80 |
| miR-24 | 349.70 | 504.78 | 141.20 | 408.89 |

Total 1031 miRNAs were identified and 369 miRNAs were expressed in all samples.

The top 20 most abundant miRNAs of total miRNA expression in bovine blood plasma are listed here

- bta-miR-286
- bta-miR423-5p
- bta-miR-191
- bta-miR-25
- bta-miR-92a
- bta-miR-30d
- bta-miR-148a
- bta-miR-186
- bta-miR-27b
- bta-miR-30e-5p
- bta-miR-151-3p
- bta-miR-146b
- bta-miR-26a
- bta-miR-660
- bta-miR-6529a

- bta-miR-150
- bta-miR-30a-5p
- bta-miR-451
- bta-let-7i
- bta-let-7c

Among miRNAs identified in plasma in all samples sequenced, we have found miRNAs involved in either myoblast proliferation or myocyte differentiation. (Table 1)

**Table 1**Expression (reads in million) of three myo-miRNAs and other miRNAs based on NGS results during four periods of muscle growth. Arrow shows the higher expression of a miRNA at a particular period of growth.

Based on the expression profile of different miRNAs, validation will be done Real time qPCR.

All these miRNAs are known to be involved in skeletal muscle growth and differentiation processes. MyomiRNA such asmiR-1 and miR-206have specific roles in modulating bovine skeletal muscle satellite cell proliferation and differentiationthrough the positive regulation of Pax7 andthe downregulation of HDAC4 (Dai *et al*, 2016) High expression levels of miR-26a are required to promote differentiation of satellitecells (Wang et al., 2018).The miR-27 family influences many cellular processes and plays an important role in down regulation of Myostatin, a negative regulator of muscle growth, thus regulation of post-natal myogenesis and promotes skeletal muscle satellite cell proliferationin animal production species (Huang et al, 2012;Zhang et al, 2018).

**CONCLUSION**

MiRNAs are necessary for skeletal muscle development and have regulatory roles throughout growth and differentiation. Little is known about role of ci-miRNA in cell-to-cell communication to regulate normal skeletal muscle processessuch as the regulation ofmuscle differentiation. Nevertheless, to our knowledge, no studies have examined ci-miRNA expression profiles during different growth periods in bovine. Thus, the primary objective of this study was to identify if a panel of miRNA-centered on regulation in the context of bovine skeletal muscle cell growth and differentiation was represented.

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