Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE MEDICHE, CLINICHE E SPERIMENTALI
INDIRIZZO: NEUROSCIENZE
CICLO XXVII

Detection of circulating miRNAs in serum in a mouse model of Collagen VI deficiency

Direttore della Scuola : Ch.mo Prof. Gaetano Thiene

Coordinatore d'indirizzo: Ch.mo Prof.ssa Elena Pegoraro

Supervisore : Ch.mo Prof.ssa Elena Pegoraro

Dottorando : Francesco Catapano

Summary

Collagen VI is the main extracellular matrix (ECM) protein synthesized and secreted mainly by fibroblasts that forms a structurally network of microfilaments.
Collagen VI binds several cell surface receptors and ECM components, suggesting that collagen VI is an important mechanical link between muscle cells and surrounding ECM. [1]
Mutations related to *COL6A1*, *COL6A2* and *COL6A3* genes encoding for the three main collagen VI chains, cause the collagen VI-related myopathy, a specific subtype of congenital muscular dystrophy (CMD).
In particular Ullrich congenital muscular dystrophy (UCMD) is a severe form characterized by progressive muscle wasting leading to loss of ambulation around ten years of age and respiratory problems in late childhood or young adulthood.
Bethlem Miopathy (BM) is a mild-form characterized by a slow course usually without respiratory symptoms where around 50% of patients lose ambulation in the fifth decade. [2]
Being the genetic test aimed to find mutations in *COL6A* genes expensive and time consuming mainly because the large size of the three genes, (106 coding exons in total [2]) the diagnosis of collagen VI-related myopathies is based on the clinical signs assessment coupled to the muscular biopsy which is useful to highlight any dystrophic or myopathic changes in the muscle fibers of the patient.
The aim of the present work has been to identify a panel of miRNAs significantly dysregulated in *col6a1*$^{-/-}$ mice, the animal model of Bethlem myopathy and Ullrich congenital muscular dystrophy.
miRNAs represent an ideal biomarker because they are easy to detect, well preserved in serum and non invasive.
The findings of this work can be translated, in a further step, in a cohort of affected patients in order to become a powerful tool for the diagnosis and the follow-up of collagen VI-related myopathy.

Research projects aimed to link the differential miRNA expression to pathological conditions are increasing and this suggests how miRNAs could be used as powerful tool to track disease related changes on a large scale in the forthcoming years.

In order to identify differentially expressed miRNAs in knock-out *col6a1*$^{-/-}$ (KO) mice serum, a miRNAs expression profiling analysis by SYBR Green-based real-time PCR of 752 miRNAs in 5 *col6a1*$^{+/+}$(WT) and 6 *col6a1*$^{-/-}$ has been performed. Fifty one (6.5%) of the 333 miRNAs expressed in *col6a1*$^{-/-}$ mice serum, showed a significant dysregulation between *col6a1*$^{-/-}$ and WT mice serum.

The statistical analysis revealed 17/51 miRNAs up-regulated and 34/51 down-regulated.

To further characterized the dysregulated miRNAs we checked their involvement in disease related pathways coupled with literature analysis.

These analyses showed that mmu-miR-195a-5p, mmu-miR-26a-5p, mmu-let-7c-5p and mmu-let-7b-5p are up-regulated in *col6a1*$^{-/-}$ mice serum, whereas mmu-miR-29b-3p and mmu-miR-29a-3p are downregulated of. These observations are quite interesting because these miRNAs could play an important role in the collagen VI-related myopathies linking genes involved in essential signalling pathway for skeletal muscle cells development, differentiation or coding for ECM proteins.

In particular miRNAs targets interfere with ERBb, MAPK, PIK3/Akt, mTOR, ECM-receptors, actin cytoskeleton, focal adhesion and calcium signalling.

In conclusion, this project has identified 6 dysregulated microRNAs (mmu-miR-195a-5p, mmu-miR-26a-5p, mmu-let-7c-5p, mmu-let-7b-5p, mmu-miR-29b-3p and mmu-miR-29a-3p) that could be used in future studies as biomarkers and possibly as a tool for the diagnosis and the follow-up of collagen VI-related myopathies.

Further analyses in larger cohorts of BM and UCMD patients are needed in order to validate the data obtained in mice models.

Riassunto

Il collagene VI, sintetizzato e secreto principalmente da fibroblasti, è uno dei maggiori costituenti della matrice extracellulare (ECM) ove forma una fitta rete di microfilamenti.
Questa proteina prende contatto con diversi recettori localizzati sulla membrana cellulare e allo stesso tempo con altri componenti della matrice extracellulare indicando che, probabilmente, il collagene VI ha un ruolo importante nella trasmissione dei segnali tra l'interno e l'esterno della cellula oltre che una funzione puramente strutturale.[1]
Mutazioni legate ai geni *COL6A1, COL6A2* e *COL6A3,* codificanti per le catene principali del collagene VI, causano la distrofia muscolare congenita di Ullrich (UCMD) e la variante mite, miopatia di Bethlem (BM).
La UCMD è una forma severa di distrofia muscolare caratterizzata da un deficit di forza progressivo che porta, nella maggior parte dei pazienti, alla perdita della deambulazione attorno al decimo anno di vita e a problemi respiratori nella tarda adolescenza o all'inizio dell'età adulta.
La BM è una forma di miopatia meno severa rispetto alla UCMD caratterizzata da un decorso più lento e dalla assenza di insufficienza respiratoria. Circa il 50% dei pazienti con BM perde la deambulazione dopo la quinta decade. [2]
La ricerca di mutazioni nei geni *COL6A1, COL6A2* e *COL6A3* è una procedura costosa e complessa (la sequenza codificante dei tre geni è pari a 106 esoni [2]), e la diagnosi di collagenopatia è spesso basata sulla presentazione clinica e sulle caratteristiche della biopsia muscolare.
La biopsia muscolare è una procedura invasiva di elevato costo.
Lo scopo di questo studio è stato quello di identificare dei microRNA (miRNA) significativamente disregolati nel siero del topo *col6a1*$^{-/-}$, modello animale di collagenopatia, per individuare un pannello di biomarcatori da poter studiare, in una fase successiva, in pazienti affetti da UCMD e BM.
I miRNA sono dei biomarcatori ideali, non invasivi, in quanto facili da dosare e stabili nel siero.
L'identificazione di biomarcatori non invasivi rappresenterà un importante strumento per la diagnosi e il follow-up delle collagenopatie.

Al fine di identificare miRNA differenzialmente espressi nel siero del topo *col6a1*$^{-/-}$ (KO) e *col6a*$^{+/+}$ (WT), sono stati analizzati 752 diversi miRNA in 11 campioni (rispettivamente n=5 WT e n=6 KO).

333 miRNA sono risultati espressi nel siero dei topi KO ed il 6,5%, 51 miRNA, sono risultati significativamente disregolati nel siero dei topi WT rispetto a quello dei KO (17/51 miRNAs up-regolati e 34/51 down-regolati).

I miRNA disregolati sono stati caratterizzati avvalendosi di specifiche analisi di pathway accoppiate ai dati trovati in letteratura.

I miRNAs: mmu-miR-195a-5p, mmu-miR-26a-5p, mmu-let-7c-5p, mmu-let-7b-5p sono risultati up-regulati nel siero dei topi *Col6a1*$^{-/-}$ mentre mmu-miR-29b-3p e mmu-miR-29a-3p down-regulati.

Questi miRNAs potrebbero giocare un ruolo importante nella patogenesi delle collagenopatie in quanto legano geni coinvolti in importanti pathway molecolari necessarie ad un corretto sviluppo e differenziamento della cellula muscolare, e a pathway coinvolti nella produzione di proteine della ECM.

I pathway interessati sono stati: ERBb, MAPK, PIK3/Akt, mTOR, ECM-receptors, actin cytoskeleton, focal adhesion e calcium signalling.

In conclusione in questo studio sono stati identificati e selezionati 6 miRNA significativamente disregolati (mmu-miR-195a-5p, mmu-miR-26a-5p, mmu-let-7c-5p, mmu-let-7b-5p, mmu-miR-29b-3p and mmu-miR-29a-3p) nel modello murino di topo knock down per il collagene VI.

Questi miRNA sono candidati alla caratterizzazione nel siero di pazienti con UCM e BM e potrebbero diventare ottimi biomarcatori non invasivi per la diagnosi ed il follow-up dei pazienti.

Ulteriori studi verranno effettuati in ampie coorti di pazienti affetti al per confermare questi dati preliminari.

1.Introduction

1.1 Muscular dystrophies

Muscular dystrophies (MDs) are a clinically and genetically heterogeneous group of diseases sharing clinical signs and symptoms, in particular a variable degree of progressive muscle weakness that affects limb, facials and axial muscles associated to dystrophic pathological features observed in muscle biopsy [3]. The age of onset, severity and progression rate is variable between patients and it depends on the type of MD [4].

Dystrophic muscle is usually characterized by alteration in muscle fibers size with infiltration of connective and fatty tissues, and centrally located nuclei. Often, the cell membrane is fragile and subjects to extensive damage leading to necrosis and muscle wasting [5].

Nowadays, a better understanding of the molecular pathway underlying muscular dystrophies, combined to new treatment approaches, changed both the natural history and life of the patients in terms of quality and lifespan. Several studies identified some of the mutant genes leading to MD disorders and allowing the development of new clinical trials.[6, 7]

1.1.2 Classification and main clinical manifestations

MDs are classified based on clinical and genetic features.

DMD is the main type of MD affecting 1 per 3600-6000 live male births; other muscular disorders are the facioscapulohumeral muscular dystrophy (FSHD) (1 per 20000 live birth), congenital muscular dystrophy (CMD) (from 1 per 21500 to 1 per 16000 live birth) and the limb-girdle muscular dystrophy (LGMD) (0.8 per 100000 live birth).

MD types differs among them in terms of onset age, pattern of inheritance weakness distribution, rate of progression, grade of hypertrophy and, in some cases by serum levels of sarcoplasmic enzymes like creatine kinase (CK).[8, 9]

Generally, the onset of clinical signs varies, ranging from birth or childhood to adulthood and the most consistent finding is the weakness of the skeletal muscles often associated with either muscle atrophy, hypertrophy, or both. The progression of the disease is variable and related to the gene mutations. Cardiac involvement and respiratory impairment are common features in many MDs (with different grade of severity), while a functional or structural brain involvement occurs just in a few types of congenital muscular dystrophies and rarely in limb girdle muscular dystrophy variants.[4]

	Type	Inheritance	Gene	Protein
Duchenne or Becker muscular dystrophy		X-R	DMD	Dystrophin
Limb girdle muscular dystrophy				
	Type 1A	AD	MYOT	Myotilin
	Type 1B	AD	LMNA	Lamin A/C
	Type 1C	AD	CAV3	Caveolin-3
	Type 1D	AD	DNAJB6	Co-chaperone DNAJB6
	Type 1E	AD	DES	Desmin
	Type 1F	AD	Unknown	Unknown
	Type 1G	AD	Unknown	Unknown
	Type 1H	AD	Unknown	Unknown
	Type 2A	AR	CAPN3	Calpain-3
	Type 2B	AR	DYSF	Dysferlin
	Type 2C	AR	SGCG	γ-sarcoglycan
	Type 2D	AR	SGCA	α-sarcoglycan
	Type 2E	AR	SGCB	β-sarcoglycan
	Type 2F	AR	SGCD	δ-sarcoglycan
	Type 2G	AR	TCAP	Titin cap (telethonin)
	Type 2H	AR	TRIM32	Tripartite motif-containing 32 (ubiquitin ligase)
	Type 2I	AR	FKRP	Fukutin-related protein
	Type 2J	AR	TTN	Titin
	Type 2K	AR	POMT1	Protein-1-O-mannosyl-transferase 1
	Type 2L	AR	ANO5	Anoctamin 5
	Type 2M	AR	FKTN	Fukutin
	Type 2N	AR	POMT2	Protein-O-mannosyl-transferase 2
	Type 2O	AR	POMGNT1	Protein-O-linked mannose β 1,2-N-
	Type 2P	AR	DAG1	Dystrophin-associated glycoprotein 1
	Type 2Q	AR	PLEC1	Plectin 1
Facioscapulohumeral muscular dystrophy				
	Type 1	AD	Unknown	DUX4 and chromatin rearrangement
	Type 2	AD	Unknown	SMCHD1
Emery-Dreifuss muscular dystrophy				
	X-linked type 1	X-R	EMD	Emerin
	X-linked type 2	X-R	FHL1	Four and a half LIM domain 1
	Autosomal dominant	AD	LMNA	Lamin A/C
	Autosomal recessive	AR	LMNA	Lamin A/C
	With nesprin-1 defect	AD	SYNE1	Spectrin repeat containing, nuclear envelope 1 (nesprin-1)
	With nesprin-2 defect	AD	SYNE2	Spectrin repeat containing, nuclear envelope 2 (nesprin-2)

Congenital muscular dystrophy with merosin deficiency (MDC1A)		AR	LAMA2	Laminin α2 chain of merosin
Congenital muscular dystrophy		AR	Unknown	Unknown
Congenital muscular dystrophy and abnormal glycosylation of dystroglycan (MDC1C)		AR	FKRP	Fukutin-related protein
Congenital muscular dystrophy and abnormal glycosylation of dystroglycan (MDC1D)		AR	LARGE	Like-glycosyl transferase
Fukuyama congenital muscular dystrophy		AR	FCMD	Fukutin
Walker–Warburg syndrome				
	With fukutin defect	AR	FCMD	Fukutin
	With protein-O-mannosyl-transferase 1 defect	AR	POMT1	Protein-1-O-mannosyl-transferase 1
	With protein-O-mannosyl-transferase 2 defect	AR	POMT2	Protein-O-mannosyl-transferase 2
	With protein-O-linked mannose β 1,2-N-aminyltransferase 1 defect	AR	POMGNT1	Protein-O-linked mannose β 1,2-N-aminyltransferase 1
	With fukutin-related protein defect	AR	FKRP	Fukutin-related protein
Muscle-eye-brain disease				
	With protein-O-linked mannose β 1,2-N-aminyltransferase 1 defect	AR	POMGNT1	Protein-O-linked mannose β 1,2-N-aminyltransferase 1
	With fukutin-related protein defect	AR	FKRP	Fukutin-related protein
	With protein-O-mannosyl-transferase 2 defect	AR	POMT2	Protein-O-mannosyl-transferase 2
Congenital muscular dystrophy due to glycosylation disorder		AR	DPM2	Dolichyl-phosphate mannosyltransferase polypeptide 2
Congenital muscular dystrophy due to glycosylation disorder		AR	DPM3	Dolichyl-phosphate mannosyltransferase polypeptide 3
Congenital muscular dystrophy with mitochondrial structural abnormalities		mtDNA	CHKB	Choline kinase
Congenital muscular dystrophy with rigid spine syndrome		AR	SEPN1	Selenoprotein N1
Ullrich syndrome				
	With collagen type VI subunit α1 defect	AR	COL6A1	Collagen type VI, subunit α1
	With collagen type VI subunit α2 defect	AR	COL6A2	Collagen type VI, subunit α2
	With collagen type VI subunit α3 defect	AR	COL6A3	Collagen type VI, subunit α3
Congenital muscular dystrophy with integrin α7 defect		AR	ITGA7	Integrin α7
Congenital muscular dystrophy with integrin α9 defect		AR	ITGA9	Integrin α9
Muscular dystrophy with generalised lipodystrophy		AR	PTRF	Polymerase I and transcript release factor (cavin-1)
Oculopharyngeal muscular dystrophy		AD or AR	PABPN1	Polyadenylate binding protein nuclear 1

Imagine 1. Classification of muscular dystrophies. X-R=X-linked recessive. OMIM=Online Mendelian Inheritance in Man. AD=autosomal dominant. AR=autosomal recessive. (Mercuri, E. and F. Muntoni, *Muscular dystrophies.* Lancet, 2013.)

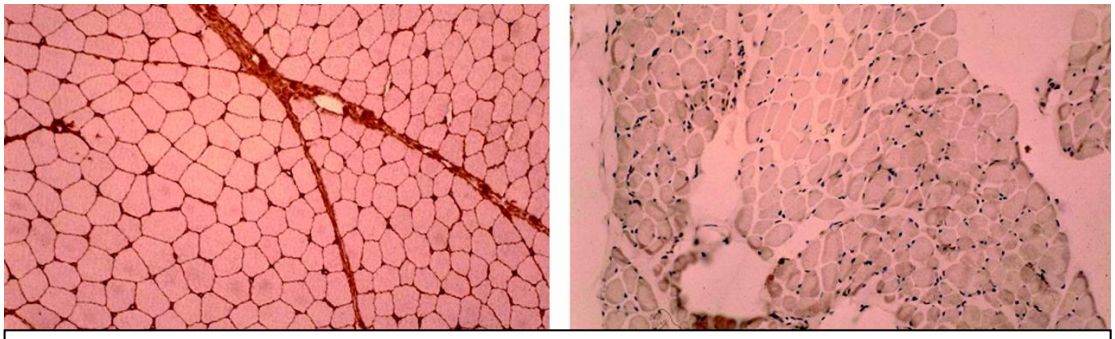
1.1.3 Diagnosis

In order to ameliorate the life quality and disease history an early diagnosis is essential.

Usually it is made by an assessment of clinical signs and symptoms coupled with genetics and biochemical analyses aimed to characterize the MD type.

Muscle biopsy is usually mandatory to assess the diagnosis and gives important information regarding the fibers morphology, the presence/absence of inflammatory infiltrates or fatty tissue and it is essential to exclude overlapping features observed during the diagnosis assessment in many MD patients.

As mentioned before, serum creatinine kinase (CK) concentrations are often higher than normal values, but do not indicate a specific MD [4].



Imagine 2. Immunostaining of collagen VI in skeletal muscle showing that collagen VI is absent in basement membrane muscle fibers of a UCMD patient (right) compared to a normal control (left). (Imagine taken from Lampe A K , and Bushby K M D J Med Genet 2005;42:673-685)

1.2 Congenital muscular dystrophies

Congenital muscular dystrophies (CMDs) are a group of hereditary myopathies clinically and genetically heterogeneous mainly characterized by autosomal recessive inheritance having variable clinical course and presenting sometimes central nervous system (CNS) and ocular symptoms.

In general CMDs biopsies highlight variability in the size of muscle fibers which show internal nuclei, especially during the early stages of the disease, and infiltrations of adipose tissue.

CMDs classification has been proposed and continuously updated, but in general they are divided into 5 subtypes based on the phenotypic and genotypic features [10].

- Forms of CMDs caused by mutations in genes encoding for basal lamina structural proteins or extracellular matrix (ECM) proteins.
  It has been reported CMDs variants due to mutations in *COL6A1*, *COL6A2* and *COL6A3* genes such as the Ullrich congenithal muscular dystropy (UCMD) and the Bethlem myopathy (BM) or CMD variants caused by mutations occuring in laminin α-2 (*LAMA2*), integrin α-7 (*ITGA7*) and integrin α-9 (*ITGA9*) genes
- CMD forms could be linked to genetic mutations in glycosyltransferases that affect the glycosilation of α-dystroglycan (ADG). Several genes involved in this pathway could lead to secondary dystroglycanopathies such as Fukuyama CMD, muscle-eye-brain disease (MEB), Walker-Warburg syndrome, CMD variant 1C (MDC1C) and variant 1D (MDC1D).
- Other forms caused by deficiency of nuclear envelope proteins (LMNA and nesprin) or other proteins with unknown function localized in the endoplasmic reticulum and, finally, CMDs with mitochondrial structural abnormalities (CMDmt) [11].

1.3 Collagen VI

Collagen VI is the main extracellular matrix (ECM) protein forming a structurally network of beaded microfilaments.
It is synthesized and secreted by fibroblasts and muscle cells.

Collagen VI is mainly expressed in connective tissues [12] and is composed by three major chains: α1(VI), α2(VI) and α3(VI), encoded respectively by *COL6A1*, *COL6A2* and *COL6A3* genes.

The α1 (VI) and α2 (VI) chains are around 1000 amino acids long and 140 kDa, while α3 (VI) chain is 250-300 kDa and it is composed from a sequence of 2500-3100 amino acids. [13, 14] The three "minor" chains α4, α5 and α6 are highly homologous to the α3 chain [15, 16] .

Structurally all collagen VI chains consist of a short triple-helical region whereas the N- and C-terminal of the protein present globular structures. These globular features are largely formed by Von Willebrand factor A (VWA) consisting of around 200 amino acid residues that are assuming a Rossmann folding with a core β sheet conformation surrounded by amphipathic α helices. VWAs are present in many proteins like integrins, matrilins and collagens[17, 18] and often contain an highly conserved metal-ion-dependent adhesion site (MIDAS). Usually this motif is involved in ligand binding [19].

In collagen VI, the N terminal of α1 and α2 chains present one VWA domain each, while α3, α4, α5 and α6 chains show from seven to ten VWA domains at the N-terminal of the protein

In the C terminal region, all the chains are composed by two VWA domains, except the α5 chain which has three VWA domains.

The assembling of collagen subunits takes place in several steps.

At the beginning α1, α2 and α3 chains form triple helical monomers that are going to be arranged into disulfide-bonded antiparallel dimers.

Subsequently, the dimers are aligned and assume the tertiary protein structure of 145 nm tetramers stabilized by disulfide bonds.

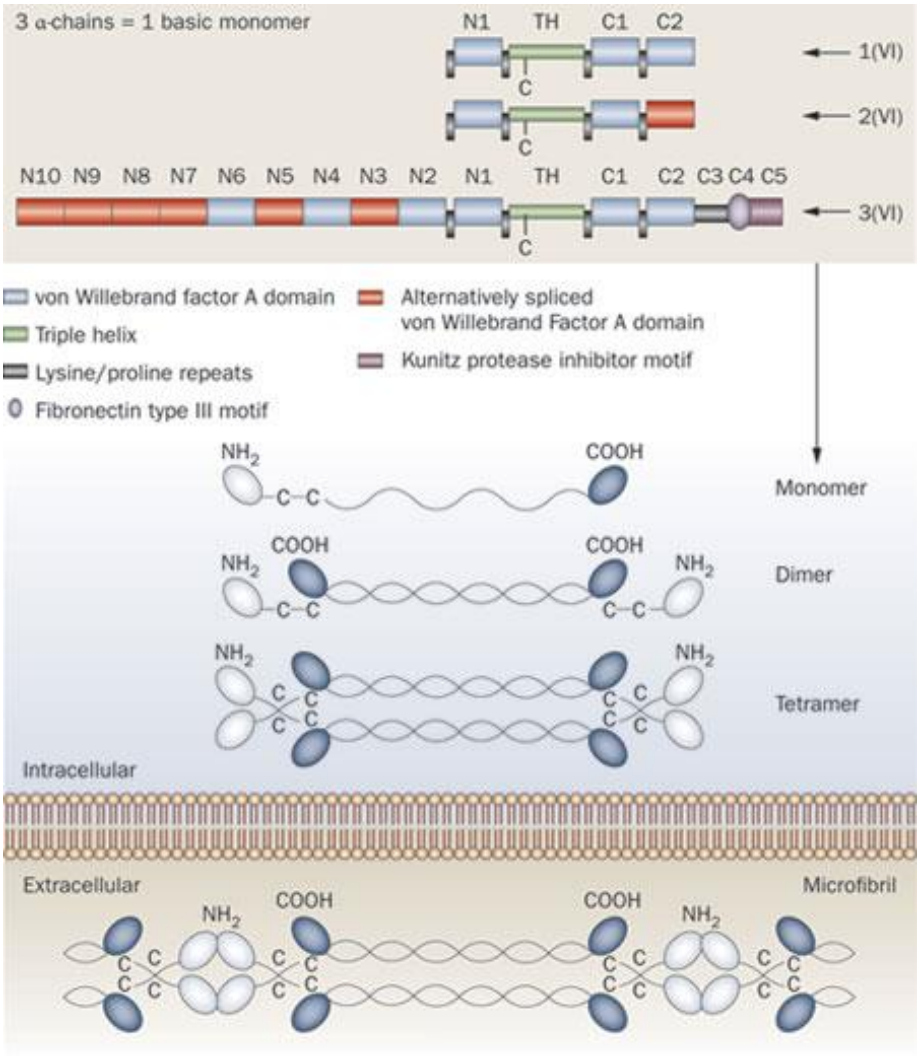
After secretion, collagen microfibrils assume a "beads-on-a-string" appearance due to the tetramers head-to-head interaction.[12]

Secreted collagen VI consists of at least 60 tetramer units up to 6 μm in length. [20]

Collagen VI is involved in interactions with cell surface receptors.

In particular, through the triple helical region and the VWA domains, collagen VI binds several cell surface receptors and especially ECM components such as integrins, fibrillar collagens, basal lamina (type IV) collagen, decorin, byglycan and fibronectin [21, 22].

These interactions with several ECM components suggest that collagen VI plays an important mechanical role between muscle cells and surrounding ECM components [1].



Imagine 3. Domains and assembly of collagen VI protein. (Imagine taken from Bönnemann, C. G. (2011) The collagen VI-related myopathies: muscle meets its matrix *Nat. Rev. Neurol.* doi:10.1038/nrneurol.2011.81)

The human *COL6A1* and *COL6A2* genes are clustering in the most distal part of chromosome 21 while *COL6A3* gene is in chromosome 2 [20].

Gene	Locus	
COL6A1	Human	21q22.3
	Mouse	chromosome 10
COL6A2	Human	21q22.3
	Mouse	chromosome 10
COL6A3	Human	2q37
	Mouse	chromosome1

Imagine 4. The loci of the genes coding for the three polypeptide chains of collagen VI protein.

1.3.2 Collagen VI gene mutation and related myopathies

Mutations affecting the three major collagen VI genes *COL6A1, COL6A2* and *COL6A3* have been associated to the congenital muscular dystrophy type Ullrich (UCMD) showing a severe phenotype, with the mild-phenotype Bethlem myopathy (BM) and with the recently identified congenital myosclerosis. [23, 24]
The disease-causing mutations are often point nonsense mutations usually inherited with recessive mode, leading to premature termination codons (PTCs) or missense mutations in glycine residues in the triple helical domains resulting in a block of the triple-helix formation. [23, 24]
These type of mutations usually arise as in-frame exon skipping pattern.
Dominant de-novo mutations affecting in particular exon 16 of *COL6A3* and exon 14 of *COL6A1* lead to UCMD or BM phenotypes respectively, even if a genotype-phenotype for the collagen VI myopathies is difficult to identify [2].
UCMD is often linked to mutations in *COL6A1, COL6A2* and *COL6A3* genes and patients' phenotype is characterized by hypotonia and muscle weakness in the neonatal period often associated with kyphosis of the spine, dislocation of the hips and proximal contractures.
Most of patients show rounded face and prominent ears and their skin could show follicular hyperkeratosis and keloid formation.
Maximum motor capacity is variable across patients and some of them are unable to be ambulant.
Respiratory insufficiency appears in the first or second decade.
Bethlem myopathy is characterized by mild-progressive muscle weakness (more proximal than distal), with contractures occuring in finger flexors, elbow and ankle.

A low proportion of BM patients lose ambulation in adult life and rarely in adolescence. However they are not affected by cardiac and respiratory clinical problems.
In the past BM has been associated to dominant mutations, whereas UCMD has been linked to recessive mutations, but nowadays this distinction is not consistent. [25]
Congenital myosclerosis is a rare disorder linked to an homozygous mutation of the *COL6A2* gene resulting in reduced expression of collagen VI wherein patients muscles highlight a "woody" consistency. [24]

1.3.3 Diagnosis

Due to the lack of specific clinical and laboratory findings, the diagnosis of collagen VI-related myopathies should be made performing genetic tests aimed to analyse the collagen VI genes.
These analysis are expensive and time consuming due to the large genes size composed by 106 coding exons.[2]
In general diagnosis is achieved combining clinical features and muscle biopsy variations, even if a loss of collagen VI could be observed also in skin fibroblasts.
In BM patients, collagen VI immunostaining is almost normal or slightly reduced in muscle. Also a secondary reduction of laminin $\beta 1$ staining expression could be observed in older patients.
In UCMD patients, collagen VI immunostaining highlight a lack or strong reduction of collagen VI in endomysium and basal lamina.[26]

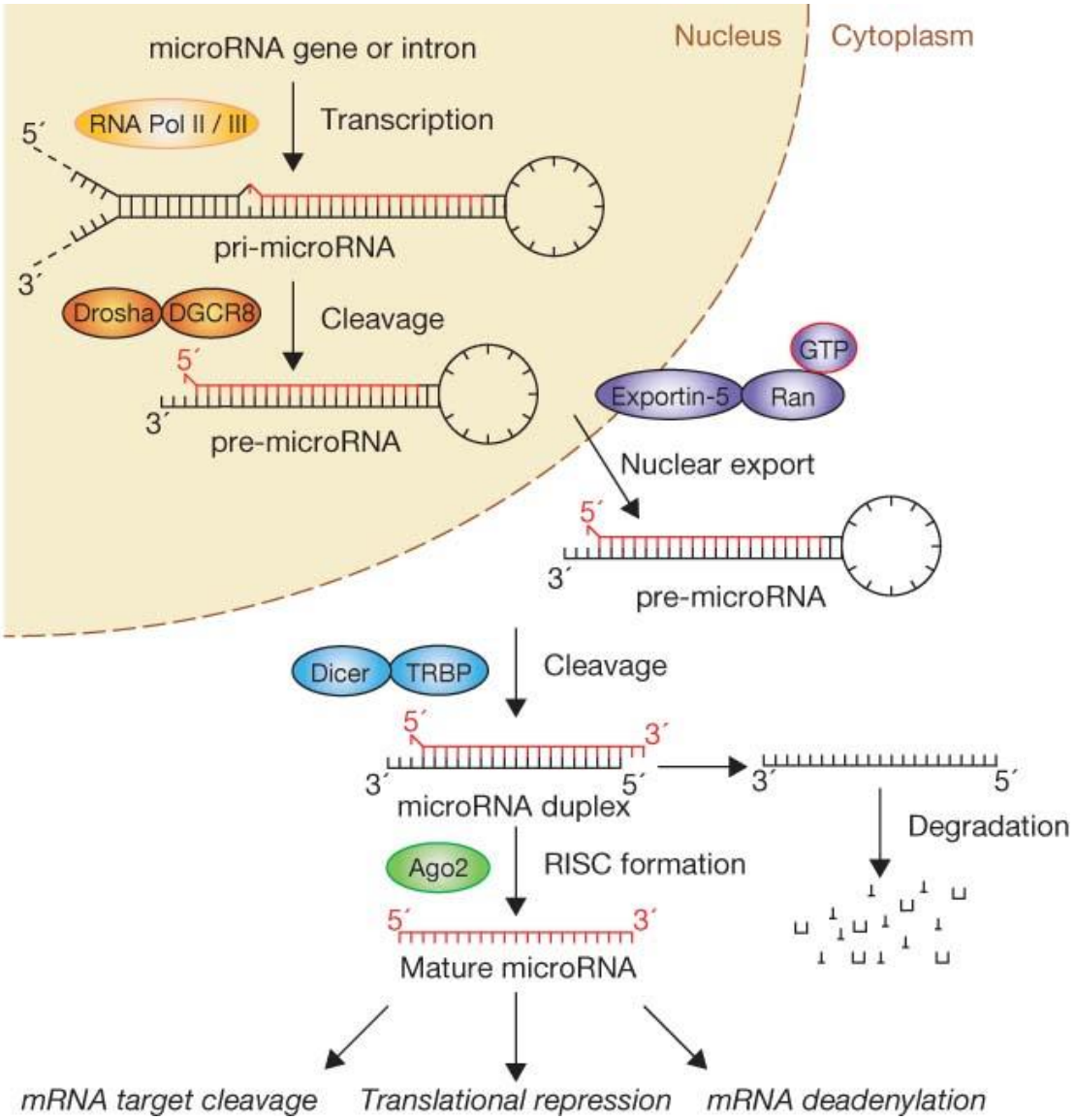
1.4 microRNAs

MicroRNAs (miRNAs) are non coding RNAs (ncRNAs) long at least 22 nucleotides that are able to bind specific mRNA targets and consequently regulate their gene expression through mRNA target degradation or, in general, inhibiting the translation.
Human genome encodes for around 1.000 miRNAs and each microRNA can bind multiple genes (hundreds).

Every single microRNA can therefore act in different molecular pathways, thus it can be of great biological interest to make predictions about the impact that the miRNAs-mRNA link may have on the disease related mechanisms. Under this perspective microRNA expression changes due to collagen VI related myopathies may also shed some light on disease pathogenesis. [27]

1.4.1 miRNA biogenesis and mechanism of action

The first miRNA biogenesis step occurs in the nucleus with the production of a primary transcript of the miRNA (pri-miRNA) composed by two or more hairpins encompassing the sequence of the mature miRNA in their stem.
Subsequently, the pri-miRNA is recognized and bound by the enzymes Drosha and DGCR8 that create one or more hairpins about 70 nucleotides long, called precursor miRNAs (pre-miRNAs), which will be exported to the cytoplasm by the enzyme Exportin 5 (XPO5).
RNaseIII enzyme Dicer recognizes the pre-miRNA and removes the hairpin loop, generating a double-strand RNA molecule (dsRNA) long about 20 bp .
Finally one strand of the dsRNA is incorporated in the RNA-inducing silencing complex (RISC) composed by the Argonaute protein 2 (AGO-2) and the GW182.
Once assembled, RISC binds the 3 'UTR of a gene having a sequence complementary to the miRNA.
This binding leads to target gene cleavage mediated by AGO-2, if there is an high homology between miRNA and target gene, or may lead to degradation due to the mRNA deadenylation.
In general, a good affinity between the two molecules requires a 6-8 continuous base pairing between target mRNA and miRNA seed region (formed by nucleotides in position 1 to 8 of the mature miRNA).[28]



Imagine 5. MicroRNA biogenesis and their repressing activity. (Imagine taken from Julia Winter, Stephanie Jung, Sarina Keller, Richard I. Gregory & Sven Diederichs – 2009 *Nature Cell Biology* 11, 228 – 234,)

1.4.2 microRNA as potential biomarkers for neuromuscular disorders

A biomarker is a measurable biological element that is able to reveal the presence of pathological status and possibly is variable in response to pharmacological treatments.
A valid biomarker would also be detectable in early disease stages, be non-invasive and its detection should be fast and relatively cheap.
In this sense microRNAs are a great example of candidate biomarkers and nowadays the research projects aimed to study the differential miRNA expression linked to pathological conditions are increasing.
Cell-free nucleic acids such as miRNAs can be detected in different body tissues and fluids like blood, where they are stably thanks to the association with the RISC complex or because they are included in microvescicles or exosomes.
Changes of miRNA expression profiles in neuromuscular diseases could be an useful biomarker for disorders like collagen VI-related diseases which require cheaper, faster and less invasive methods of diagnosis.[29]
To date, several studies have been focused to the identification of miRNAs dysregulated in skeletal muscle disorders.
Eisenberg and colleagues profiled miRNAs in muscle samples of human patients with primary muscle disorders and found a set of miRNAs commonly dysregulated in patients.[30]
Previous works have shown that members of miR-1/206 and miR-133a/133b miRNA families are specifically expressed in both skeletal and cardiac muscle.
These miRNAs originate from the same transcripts on three separate chromosomes and the myogenic transcription factors SRF, MEF2 and MyoD regulate and control the expression of miR-1 and miR-133a in muscles. [31]
Cacchiarelli and colleagues showed that microRNAs, specifically expressed in muscle cells, were released into the blood of DMD patients as a consequence of muscle degeneration and their amount correlates with the severity of the disease.
In particular miR-1, miR-133 and miR-206 were highly abundant in the bloodstream of DMD patients.
In the same work, the results obtained from patients have been confirmed in the animal model of DMD, the *mdx* mouse.
In particular in 2 month-old animals, both miR-1 and miR-206 were more abundant in the serum of *mdx* mice in comparison to WT controls.[32]

Zaharieva and colleagues showed higher amounts of miR-1, miR-133a and miR-133b levels in serum of DMD patients during the early stages of the disease and a consequently reduction of their levels related to the disease progression.
They have hypothesized that lower amounts of circulating miRNAs might reflects the loss of muscle fibres replaced by connective and adipose tissues.[33]

2.Aim

Nowadays the diagnosis of collagen VI-related myopathies and congenital muscular dystrophies in general is based on clinical signs assessment coupled to genetic analysis and muscular biopsy, which is useful to highlight any dystrophic changes in patients'muscles.

miRNAs have been defined as ideal biomarkers and are also stable in serum. My final goal in this project was the identification of a panel of candidate miRNAs

significantly dysregulated in the animal model of Bethlem myopathy and Ullrich congenital muscular dystrophy that may become candidate to be studied in affected patients.
These investigations could shed new light for the diagnosis and the follow-up of collagen VI-related myopathies.

The project has been divided into the following steps:

a) Retrotranscription and identification of miRNAs expressed in the serum of *col6a1*$^{-/-}$ and wild type mice by qPCR.

b) Identification of up-regulated and down-regulated miRNA levels comparing serum of *col6a1*$^{-/-}$ and wild type serum performing statistical analyses.

c) Selection of miRNAs significantly dysregulated in order to create a miRNA panel which could be studied in future analyses in serum of patients with Bethlem myopathy and Ullrich congenital muscular dystrophy. The candidates selection has been made through pathway analyses coupled with literature data search.

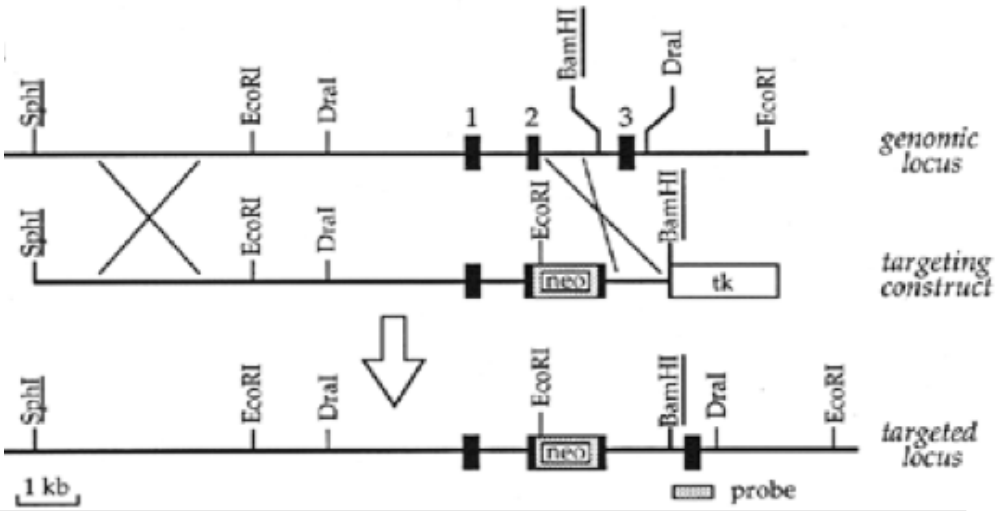
3.Material and methods

3.1.1 *Col6a1* $^{-/-}$ mutant mice

In order to study the collagen VI function, Bonaldo *et al.* generated a mouse model with inactivation of *col6a1* gene using "targeted gene disruption".

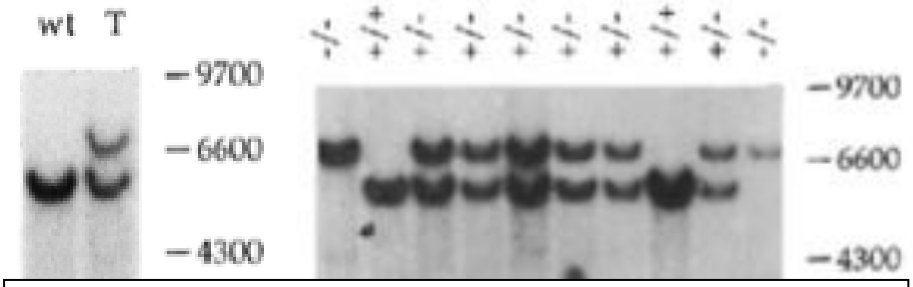
In embryonic stem cells (ES cells) the *col6a1* gene has been inactivated using a target vector containing a neomycin-resistant cassette included in the second exon.

Heterozygous mice for the mutation have been created by crossing male chimeras with C57BL/6 female.



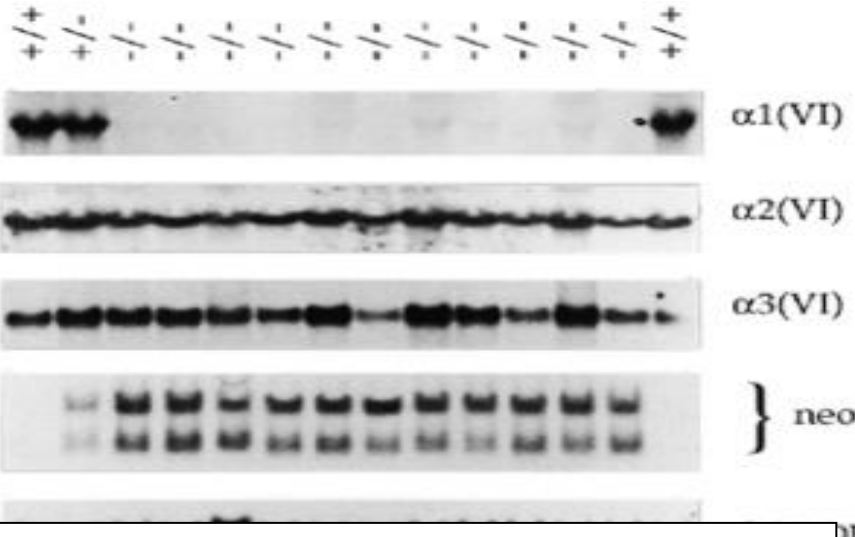
Imagine 6. Structure of the 5' col6a1 murine gene portion, structure of the construct and resulting targeted loci. (Imagine taken from Bonaldo, P., et al., *Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy.* Hum Mol Genet, 1998. 7(13): p. 2135-40.)

So of the ne e blocking the translation from amino acid 66 and increasing the DraI fragment (from 5.6 to 6.8 kb).



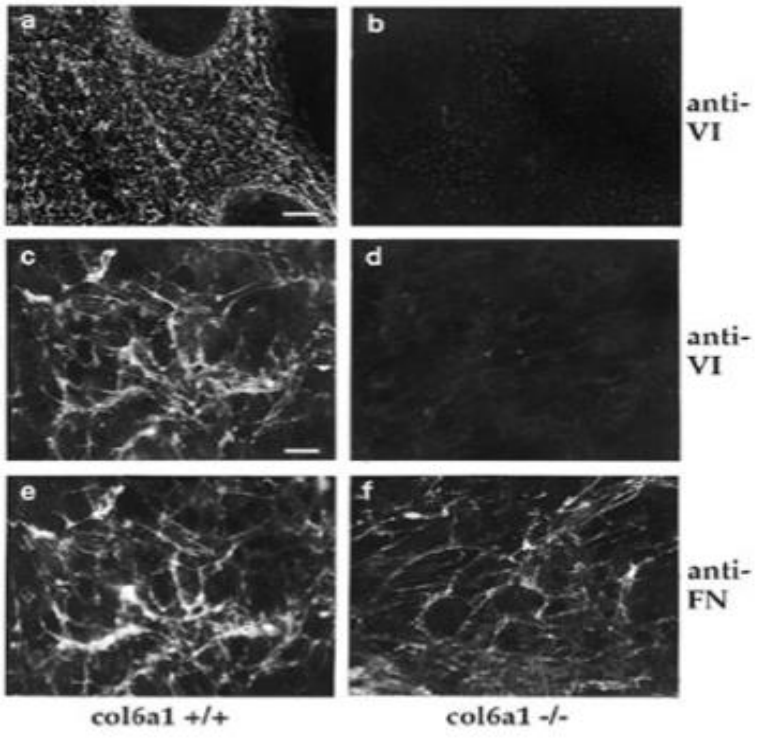
Imagine 7. Southern blot analysis of ES cells. (Imagine taken from Bonaldo, P., et al., *Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy.* Hum Mol Genet, 1998. 7(13): p. 2135-40.)

Northern blot analysis of total RNA extracted from primary embryonic fibroblasts showed how cells -/- did not express α1 (VI) mRNA, while gene expressions of α2(VI) and α3(VI) were not affected.

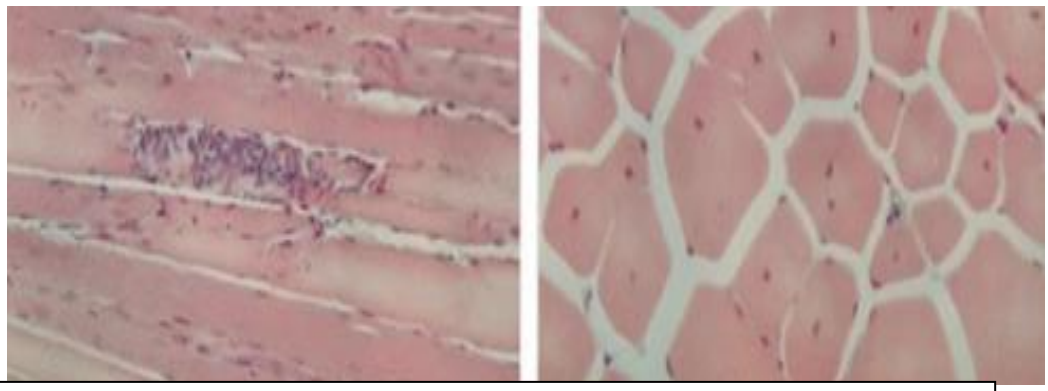


Imagine 8. Northern blot of primary embryonic fibroblasts -/- (Imagine taken from Bonaldo, P., et al., *Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy.* Hum Mol Genet, 1998. 7(13): p. 2135-40.)

The lack of the α1 (VI) chain leads to a block of collagen VI synthesis because the three chains can not be assembled.
That has been showed by collagen VI immunofluorescence analysis where it is evident an absence of collagen VI staining in the *col6a1*$^{-/-}$ embryo tissues.
In addition, histological changes in skeletal muscle of *col6a1*$^{-/-}$ mice were detected. In particular, marked signs of myopathy features as muscle necrosis, phagocytosis and variation in the fibers diameter were found.[34]



Imagine 9. Immunodetection of colVI. (a and b) sections of 16 days old WT +/+ and col6a1-/- mice thoracic wall and fibroblasts (c and d) stained with rabbit IgG against mouse collagen VI. The same fibroblasts stained with antibody against fibronectin (anti-FN). (Imagine taken from Bonaldo, P., et al., *Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy.* Hum Mol Genet, 1998. 7(13): p. 2135-40.)



Imagine 10. (Top-Left) Longitudinal section of 40 days old col6a1-/- quadriceps muscle shows necrosis and macrophage infiltration of a muscle fiber and 4 months old col6a1-/- quadriceps (Top-right) shows fibers with central nuclei. (Imagine taken from Bonaldo, P., et al., *Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy.* Hum Mol Genet, 1998. 7(13): p. 2135-40.)

3.

Animal models and related procedures have been performed at the Department of Molecular Medicine, University of Padova, Padova, Italy.

Bloods from n=5 *col6a1*$^{+/+}$ (WT) of the inbred C57BL/6J strain and n=6 *col6a1*$^{-/-}$ (KO) previously backcrossed in the C57BL/6J strain have been collected from jugular veins.

All the mice were 8 months old male.

The blood has been transferred into a 1.5 ml Eppendorf Safe-Lock Tubes™ and allowed to clot for 45' at room temperature, for 15’ on ice and later centrifuged at 3000 g x 10' at 4°C.

Finally the supernatant was collected, transferred in a 1,5 ml Eppendorf Safe-Lock Tubes™ and frozen at -80°C.

3.2 miRNA Isolation

RNA degradation issue has been removed working in an RNA free environment using RNaseZap® RNase decontamination solution (Life Technologies) to clean workspace.

Small RNA (<1000 bp) isolation has been performed using miRCURY ™ RNA Isolation Kit (Exiqon) using the following protocol:

- After thawing, samples have been centrifuged at 3000 g x 5 min to pellet any debris and insoluble components.
- For rodent samples 50 µL of serum topped up with 200 µL of RNase free water were used.
- 200 µL supernatant from previous step to new tube were transferred and 60 µL of Lysis solution (BF) added, vortexed for 5 sec and incubated for 3 min at room temperature. For downstream PCR analysis, 1 µL RNA-Spike-in template mixture was added.
- 20 µL of Protein Precipitation Solution BF were added, vortexed for 5 sec and incubated for 1 min at room temperature. Centrifuged for 3 min at 11000 x g.
- the clear supernatant was transferred into a new collection tube (2mL, with lid)
- 270 µL Isopropanol were added and vortexed 5 sec.
- a microRNA Mini Spin Column BF was placed in a collection tube and sample was loaded onto the column, incubated for 2 min at room temperature, centrifuged for 30 sec at 11000 x g. the flow was discarded through and a column was placed back into the collection tube.
- 100 µL Wash Solution BF was added to the microRNA spin column BF, centrifuged for 30 sec at 11000 x g, the flow-through was discarded and the column was placed back into the collection tube.
- 700 µL Wash Solution 2 BF were added to the microRNA spin column BF, centrifuged for 30 sec at 11000 x g. The flow-through was discarded and the column was placed back into the collection tube.

- 250 µL Wash Solution 2 BF were added to the microRNA spin column BF. Centrifuged for 2 min at 11000 x g to dry the membrane completely.
- The microRNA spin column BF was placed in a new collection tube (1,5 mL), 50 µL of RNase free water were added directly onto the membrane of the microRNA spin column BF.
- The microRNA spin column BF was incubatee for 1 min at room temperature and centrifuged for 1 min at 11000 x g.
- The purified RNA sample was stored at -20° C for a few days or a -70°C for a long term storage.

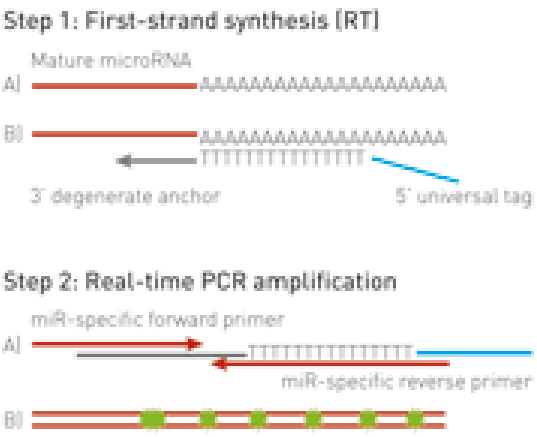
3.3 microRNA profiling

Once extracted, the microRNAs from the *col6a*$^{+/+}$ and *col6a1*$^{-/-}$ mice serum, the samples were reverse transcribed (RT) and the relative gene expression was assessed by quantitative real-time PCR using SYBR® Green, using the miRCURY LNA™ Universal RT microRNA PCR system package (Exiqon).
The kit contains four types of reagent kits:

- Universal cDNA synthesis kit II
- ExiLENT SYBR® Green master mix kit
- PCR Mouse&Rat panels including the microRNA primer sets (384 wells)
- RNA Spike-in kit

3.4 Reverse transcription

Reverse transcription reaction (RT) gave rise to a cDNA template.
During the process, a poly-a tail was added to the mature miRNA and a specific primer with a 5' universal tag was used for the synthesis.



Imagine 11. Rt-process outline (imagine taken from http://n www.exiqon.com /ls/PublishingImages/Figures/miRNA-qPCR-overview.gif)

The reverse transcription was performed using the Universal cDNA synthesis kit II and the following protocol:

- The required amount of RT working solution was prepared and placed on ice. For each sample the required mix for 2 x 384 wells plate was composed by:

Reagent	(Panel I+II) Volume (µL)
5x Reaction buffer	8,5
Nuclease-free water	18,5
Enzyme-*reverse transcriptase*	4,5
Synthetic RNA spike ins	2,5
Template total RNA (5ng/µL)	8,5
Total volume	42,5

- RT working solution was dispensed into nuclease free tubes.
- Template RNA was dispensed in each tube.
- The reaction was mixed by very gentle vortexing or pipetting to ensure that all reagents were thoroughly mixed. After mixing,the samples were spinned down.
- The samples were incubated for 60 min at 42°C.

- The *reverse transcriptase* was heat-inactivated for 5 min at 95°C.
- Immediately cooled to 4°C.
- Stored at 4°C or freeze.

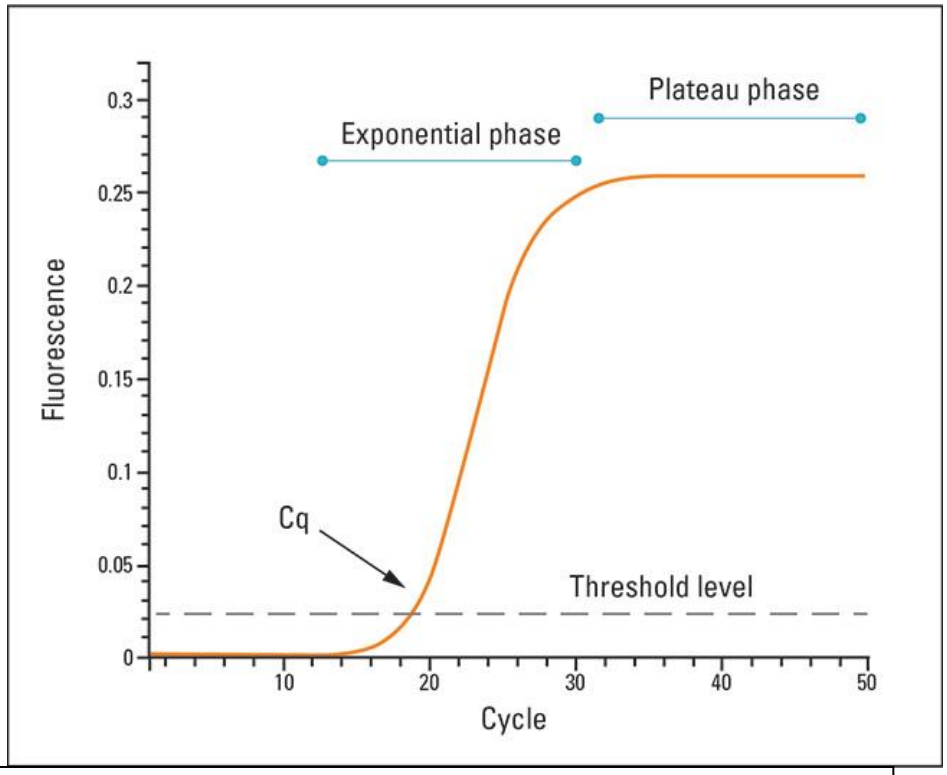
3.5 Real-Time PCR

The miRNA levels in mice serum was quantified by quantitative Real-Time polymerase chain reaction (qPCR), a technique based on the principle of a standard PCR in which a DNA exponential amplification occurs.
In qPCR this process is coupled to the DNA quantification thanks to SYBR® Green, a fluorescent reporter, added in the PCR mix.
SYBR® Green is a cyanine dyes that binds double stranded DNA.
When the amplification reaction is complete, the resulting SYBR® Green-DNA double helix complex increases the emitted fluorescence detected by Applied Biosystems 7900HT Fast Real-Time PCR System.
DNA amplification occurs through numerous cycles wherein each one is characterized by three phases:

- Denaturation phase: the DNA is incubated at high temperatures in order to allow the opening of the double helix.
- Annealing phase: the link between primers and complementary sequences occurs at a specific temperature
- Extension phase: a complementary strand to the DNA template is synthesized by the *taq polymerase* enzyme.
- Plateau phase: when there is no longer exponential amplification after a certain number of cycles in qPCR quantification of transcript occurs precisely during the exponential phase of amplification.

Several cycles of amplification are required because a given amount of product is detected.
At the end of the process the Applied Biosystems 7900HT Fast Real-Time PCR System SDS software v2.3 plotted a diagram that compared the emitted fluorescence values vs threshold cycles (Ct).

Ct (or Cq, quantification cycle) represents the point where the amount of fluorescence begins to increase rapidly because the exponential DNA duplication. The threshold level is the point in which the fluorescence is significant from background fluorescence.



Imagine 12. qPCR plot (Imagine taken from http-//www.thermoscientificbio.com/applications/pcr-and-qpcr/introduction-to-qpcr/.jpg)

The qPCR reactions were performed using the following protocol:

- The plates were spinned down in a centrifuge.
- Water, ExiLENT SYBR® Green master mix and ROX (Life technologies) reference dye were combined in order to normalize the fluorescent signal: 2000 µL 2x master mix and 1980 µL water and 80 µL ROX.
- The sample was mixed gently and spinned down.
- 42 µL cDNA (panel I+II) were added and mixed.
- 10 µL PCR Master mix were added: cDNA mixed to each well.
- The plates were sealed with optical sealing and spinned briefly.
- The used cycled conditions were:
  a)95° x 10 min.
  b)40 amplification cycles: 95°C x 10 s, 60°C x 1 min.

The Applied Biosystems 7900HT instrument Fast Real-Time PCR System was used for the analysis.
This instrument is equipped with an argon ion laser which sends light to each well of the plate in order to excite the SYBR® Green that emits light (λ=520 nm).
This process occurs every 8-10 seconds and the signal from each individual well is detected from the room and sent to the SDS software.

Imagine 13. Applied Biosystems 7900HT (imaginehttp://cgs.hku.hk/portal/files/CGS/Genomics/Realtime-PCR/7900ht_hardware.jpg)

3.6 Locked Nucleic Acid (LNA™)

The primers used are miRNA specific and optimized with LNA ™ (locked nucleic acid).
LNAs are a class of nucleic acids characterized by a specific structure in which the ribose ring is locked by a methylene bridge that creates a link between 2'-4'-O atom and the C atom.
When the LNA are incorporated into the primer, its link with the complementary DNA is faster and is more stable.
An improved affinity is confirmed by an increase in duplex melting temperatures (Tm) of about 10 ° C for each base.[35]

3.7 Quality controls and inter-plate calibration

In order to check the quality of the miRNAs isolation and cDNA synthesis processes some synthetic RNA spike-ins were added to the samples before RT and qPCR processes.
This additional step is essential because the RNA may have been degraded due to nucleases, or its quality may not be good because the presence of contaminants.
Specific spike-ins were also added to check the cDNA synthesis efficiency and the quality of its replication during the qPCR process.
Although the Applied Biosystems 7900HT Fast Real-Time PCR System is very reliable in terms of reproducibility, sometimes there may be some differences in the signal amplification for run-to-run since it is not possible to run more than one plate at a time.
In order to avoid these differences, an inter-plate calibration was performed using a specific inter-plate calibrator (IPC), which is a synthetic RNA that was added in triplicate to each plate and after qPCR analysis it must show to have a constant expression in *col6a1*$^{-/-}$ mice serum.
This has allowed a correction of the Ct, minimizing any inter-plate variations.
All the synthetic miRNA oligonucleotides and inter-plate calibrators were included in the RNA spike-in kit for quality control of the RNA isolation and cDNA synthesis steps of a miRCURY LNA™ Universal RT microRNA PCR (EXIQON).
An efficient serum profiling study requires also that red and white blood cells present membrane integrity and consequently no lysis during samples preparation or storage processing are allowed (in this case all the samples were stored at -80°C).
Given this, before proceeding to the profiling step, the hemolysis test for each sample was performed in accordance with the miRCURY ™ RNA Isolation Kit (Exiqon) protocol as described in results session.

3.8 Normalization of qPCR values

After obtaining the miRNAs expression values and made all the quality controls and the inter-plate calibration in order to minimize variations between Ct, data normalization was performed.
In general there are two strategies to normalize the qPCR data:

1) to identify a stable expressed miRNAs in serum that could be used as reference genes or, 2) the "global mean value normalization methods" described by Mestagh et al.[61]

In the Mestagh's method the global mean value of all the expressed microRNAs of each sample is used instead of the reference gene.

For both miRNA selection and normalization the following steps were undertaken:

- Exclusion of all miRNAs with undetermined value or Ct values >35 from the data analysis.
- Calculation of Ct global mean value (Ct_G) of all miRNAs expressed in each sample.
- Determination of ΔCt using the formula: $\Delta Ct = \Delta Ct_{miRNA} - \Delta Ct_G$
- Identification of Fold Changes (FC) in *col6a1*$^{-/-}$ and WT mice serum using the formula $FC = 2^{-\Delta\Delta Ct}$

All the above described analysis were performed using the spreadsheet application Microsoft Excel 2013.

3.9 Statistical analysis

In order to select the ideal test to perform for the comparison of serum levels of expressed miRNAs between KO and WT mice, first a normality test to see the data distribution (KO and WT) was performed.

The Kolmogorov-Smirnov test (KS test) was used and it showed that the values were not normally distributed.

Statistic Mann-Whitney U test was then used to compare the obtained data.

The above mentioned test is considered the non-parametric counterpart of the t-test for two independent samples and it is also called Wilcoxon rank sum test.

All the analysis were performed using the SPSS software and all P-values> 0.05 were considered significant.

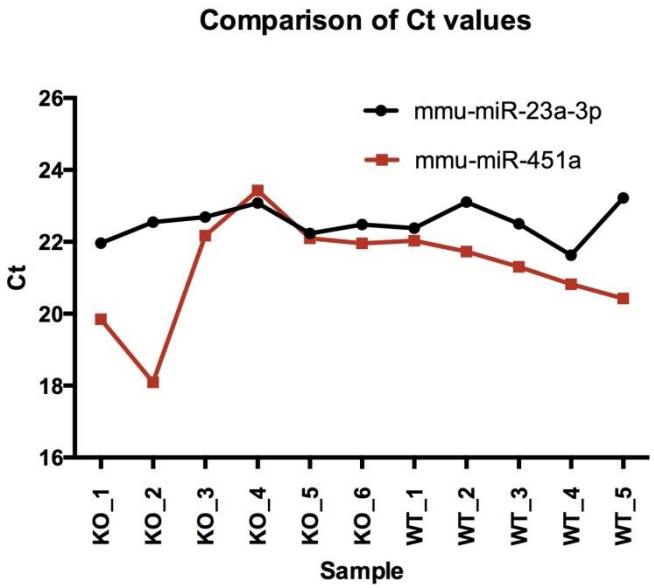
4.Results

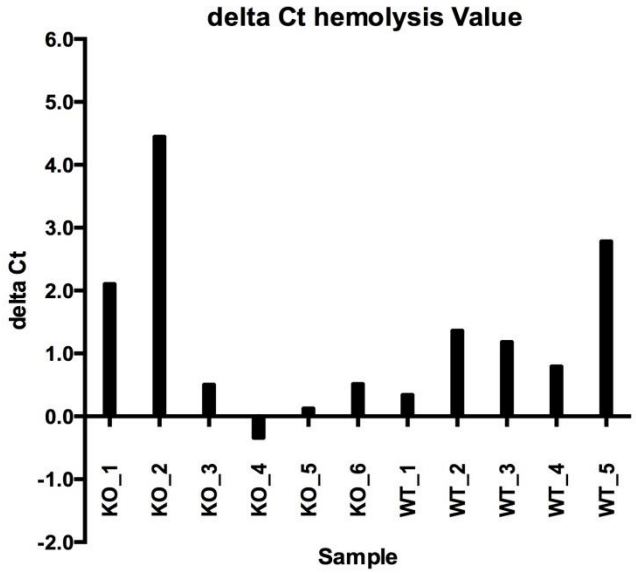
4.1 Hemolysis test of the samples

The level of a microRNA highly expressed in red blood cells (hsa-miR-451a) was compared with a specific microRNA unaffected by hemolysis (hsa-miR-23a-3p) using the following formula:

- delta Ct hemolysis Value=Ct miR-23a-3p -Ct miR-451

where values >5 are an indicator of hemolytic contamination.
In the KO and WT serum samples used for this project all the delta Ct hemolysis values were under 5 showing that samples have not been affected by erythrocyte microRNA contamination.





Imagine 14-15. Comparison of mmu-miR-23a-3p and mmu-miR-451a Ct values of KO and WT mice serum. A.Line graphs shows the Ct levels of red blood cells highly Expressed mmu-miR-451a (red) and the control mmu-miR-23a-3p unaffected by hemolysis and bar graph shows a delta Ct hemolysis Value < 0.05 for each mice serum sample.

4.2 Mice serum miRNA expression

In order to identify differentially expressed miRNAs in *col6a1*$^{-/-}$ (KO) and wild-type (WT) mice serum, miRNA expression profiles analysis by SYBR Green-based real-time PCR of n=752 miRNAs in n=11 mice (n=5 WT, n=6 Col6a1-/-) was performed.

Among the *col6a1*$^{-/-}$ mice group, n=4 were eight months old and n=2 were seven months old while all n=5 WT mice were 8 months old.

Serum miRNA levels were compared using Mann-Whitney U test and a P-value <0,05 was consider statistically significant.

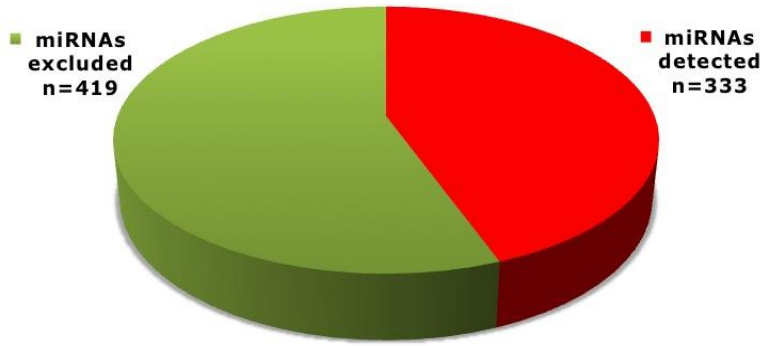
After qPCR analyses, a total of n=333 miRNA serum levels were used for statistical comparisons while n=419 miRNAs were excluded because the lack of amplification or very low expression (Ct>35) in mice serum.

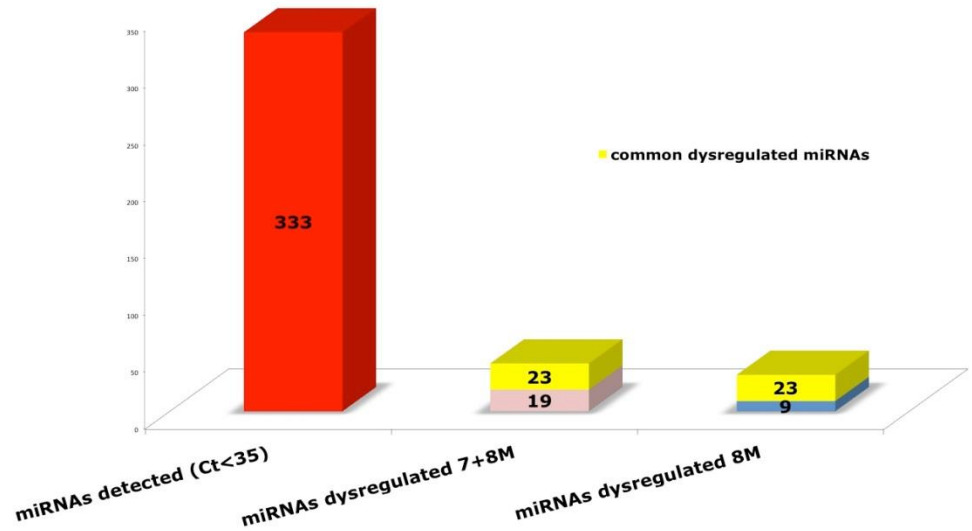
N=42 serum miRNA levels resulted significantly dysregulated after statistical analysis (P<0.05) performed in n=5 WT and n=6 KO (comparing both seven and eight months old KO mice vs WT) suggesting that expression of many miRNAs is altered in BM and UCMD.

The same statistical analysis was performed excluding n=2 *col6a1*$^{-/-}$ seven months old mice from the KO group in order to find any changes in miRNAs expression levels due to the activation of different signalling pathways in a age-dependent fashion.

Results showed that there were significant differences in serum miRNA expression between seven and eight months old mice.

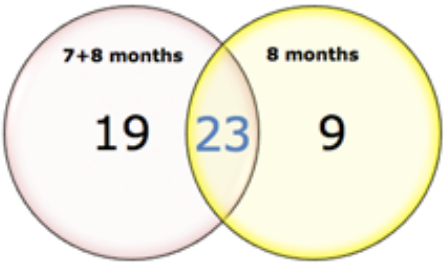
In *col6a1*$^{-/-}$ 8 old months mice serum, n=32 miRNAs were significantly dysregulated, among them n=23 dysregulated miRNAs were shared with the miRNAs dysregulated in the entire group (including seven and eight months old mice together).





Imagine 16. Pie chart showing that several miRNAs were undetected in mice serum (both WT and KO).**B** Bar graph highlight that 42 miRNAs are significantly dysregulated in 7+8 months old Col6a1-/- mice serum vs. WT (P<0,05) and 32 in 8 months old Col6a1-/- mice serum vs. WT (P<0,05). Several (22) dysregulated miRNAs of 8 months old Col6a1-/- mice are common with the miRNAs significantly dysregulated in grouped analysis (7+8 months)

All KO	Eight months KO	Common
mmu-miR-183-5p	mmu-miR-326-3p	mmu-miR-22-3p
mmu-miR-26a-5p	mmu-miR-339-5p	mmu-miR-130b-3p
mmu-let-7c-5p	mmu-miR-365-3p	mmu-miR-192-5p
rno-miR-223-3p	mmu-miR-154-5p	mmu-miR-363-3p
mmu-miR-503-5p	mmu-miR-32-5p	mmu-miR-362-3p
mmu-miR-376a-3p	mmu-miR-511-5p	mmu-miR-19a-3p
mmu-miR-30c-5p	mmu-miR-382-5p	mmu-miR-425-3p
mmu-miR-744-5p	rno-miR-667-3p	mmu-miR-29a-3p
mmu-let-7b-5p	mmu-miR-674-3p	mmu-miR-210-3p
mmu-miR-29b-3p		mmu-miR-7a-5p
mmu-miR-125a-5p		mmu-miR-215-5p
mmu-let-7f-2-3p		mmu-miR-142-5p
mmu-miR-467d-3p		mmu-miR-191-3p
mmu-miR-1983		mmu-miR-466g
mmu-miR-676-5p		mmu-miR-192-3p
mmu-miR-101a-5p		mmu-miR-130a-3p
mmu-miR-872-3p		mmu-miR-322-5p
rno-let-7f-1-3p		mmu-miR-423-3p
mmu-miR-30a-3p		mmu-miR-15a-3p
		mmu-miR-297a-5p
		mmu-miR-195a-5p
		mmu-miR-19b-3p
		mmu-miR-221-3p



Imagine 17. List of the dysregulated miRNAs in 7 and 8 months old mice.

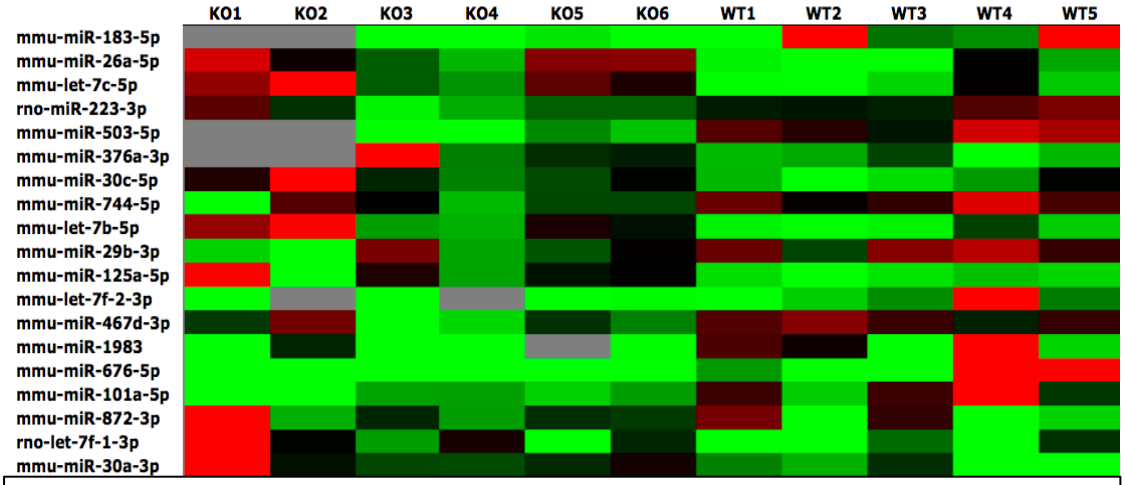
4.3 Dysregulated miRNAs expression levels

In order to better understand whether the miRNAs were significantly down or over-expressed in *col6a1*$^{-/-}$ mice serum, heat maps using the free clustering software Gene Cluster [36] were performed.

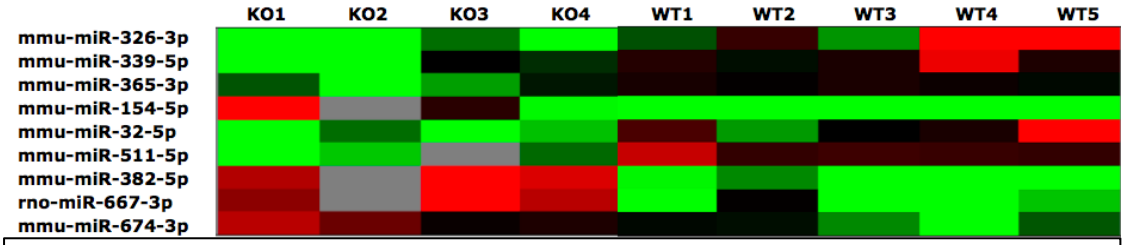
In these analysis rows represent dysregulated miRNAs and columns represent samples (KO and WT mice).

Fold changes (FC), calculated using a log2 scale (-ΔΔCt) were included in the maps where red colour represents overexpressed miRNAs and green colour represents miRNAs with lower expression.

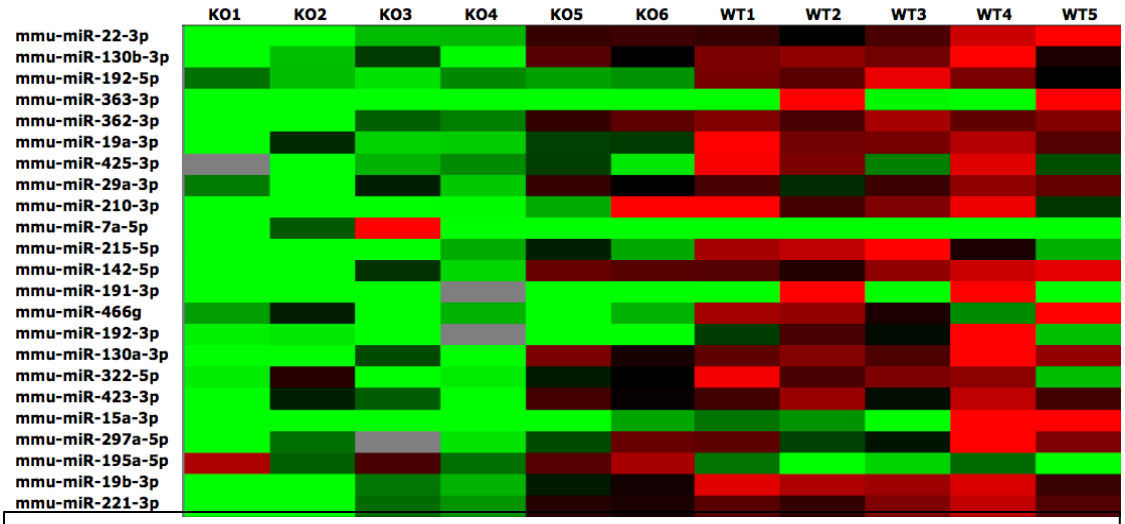
Moreover, differential heat maps were performed including and excluding seven old months *col6a1*$^{-/-}$ mice serum from the KO group and finally and heat map with common dysregulated miRNAs levels was designed.



Imagine 18. miRNA signature in Col6a1$^{-/-}$ (n=6) and in WT (n=5) mice serum.



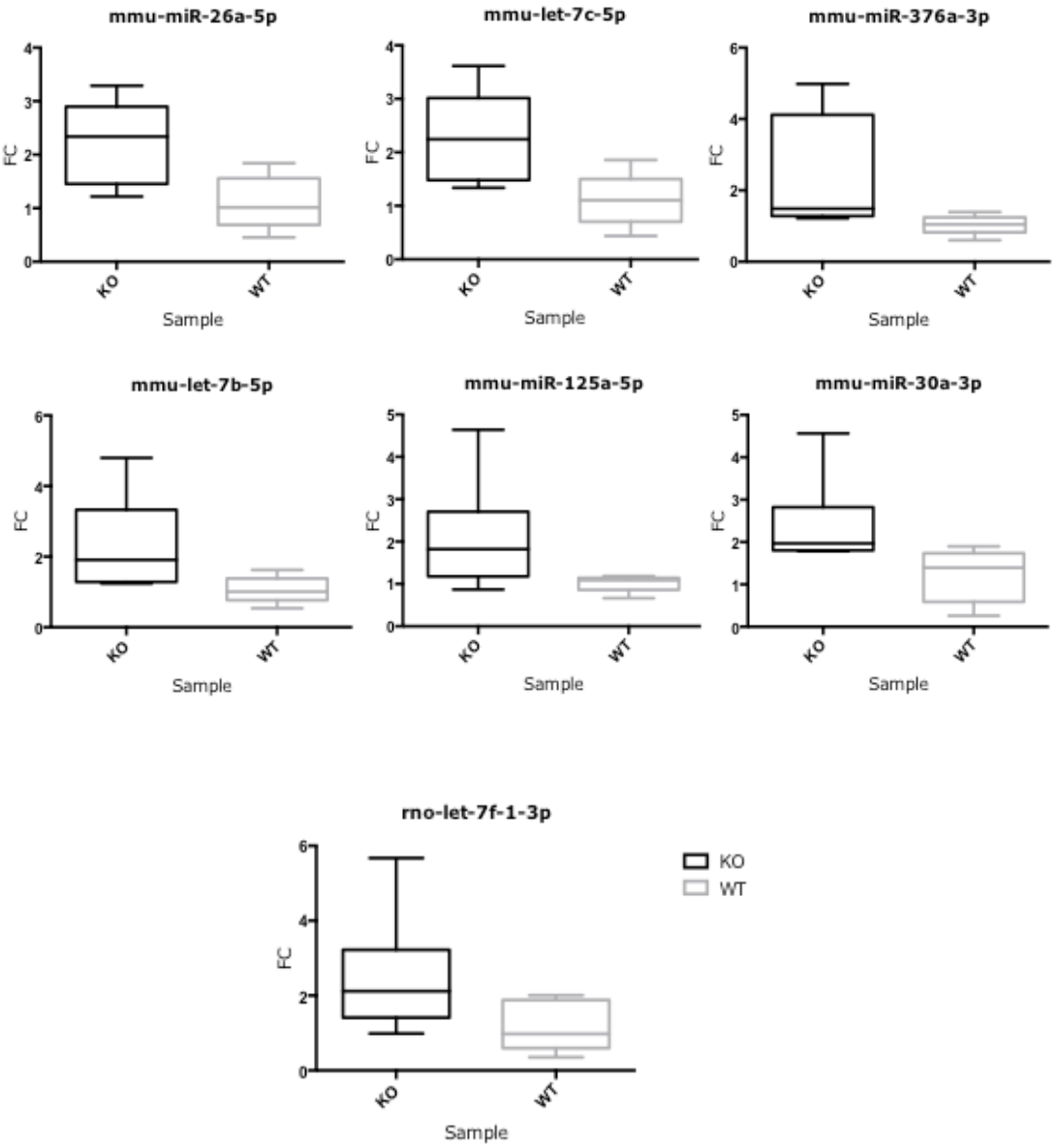
Imagine 19. miRNA signature in Col6a1$^{-/-}$ (n=4) and in WT (n=5) mice serum but not in col6a1$^{-/-}$ seven months old col6a1-/- mice serum.



Imagine 20. Heat map of common dysregulated miRNA in both groups (including and excluding seven months old col6a1$^{-/-}$ mice serum)

4.4 Dysregulated miRNAs in *col6a1*$^{-/-}$ vs. WT mice serum

Heat maps showed that there were n=9 up-regulated miRNAs in *col6a1*$^{-/-}$ mice serum (mmu-miR-26a-5p, mmu-let-7c-5p, mmu-miR-376a-3p, mmu-let-7b-5p, mmu-miR-125a-5p, rno-let-7f-1-3p, mmu-miR-30a-3p, mmu-miR-30c-5p, mmu-miR-872-3p), among them n=2 (mmu-miR-30c-5p, mmu-miR-872-3p) were only slightly up-regulated based on the FC values, and n=5 down-regulated miRNA (mmu-miR-183-5p, mmu-let-7f-2-3p, mmu-miR-1983, mmu-miR-676-5p, mmu-miR-101a-5p) and n=5 slightly down-regulated miRNAs (rno-miR-223-3p, mmu-miR-503-5p, mmu-miR-744-5p, mmu-miR-29b-3p and mmu-miR-467d-3p).

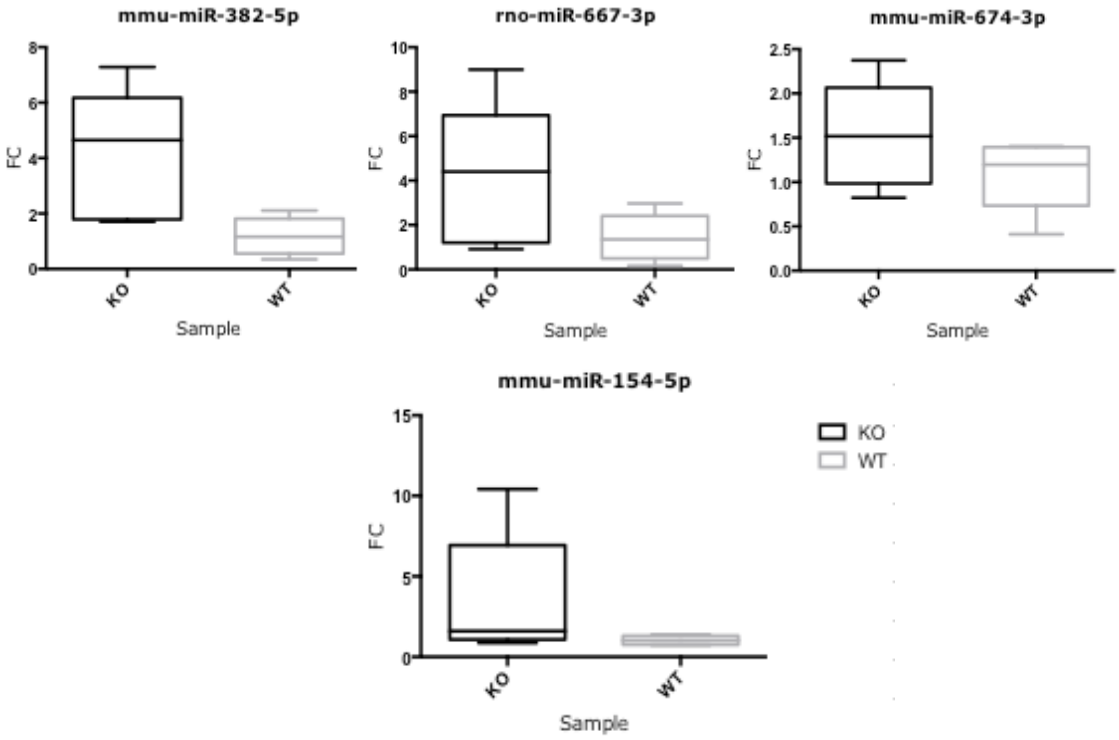


Imagine 21. Upregulated serum miRNA levels in col6a1$^{-/-}$ (n=6) vs. WT (n=5) mice serum. Data are presented as a Fold changes (FC) using a log 2 scale (-ΔΔCt). All P-Values are <0.05 (Mann-Whitney U test)

4.5 Dysregulated miRNAs in *col6a1*$^{-/-}$ 8 months old vs. WT mice serum

As already mentioned, in order to find any changes in miRNAs expression serum levels between seven and eight months old mice, a specific analysis excluding seven months old *col6a1*$^{-/-}$ mice serum from the KO group was performed.
In this case the heat map showed that n=3 miRNAs were up-regulated (mmu-miR-154-5p, mmu-miR-382-5p, rno-miR-667-3p), n=1 was slightly up-regulated

(mmu-miR-674-3p) and n=5 (mmu-miR-326-3p, mmu-miR-339-5p, mmu-miR-365-3p, mmu-miR-32-5p, mmu-miR-511-5p) were slightly down regulated in KO mice serum in comparison with WT.

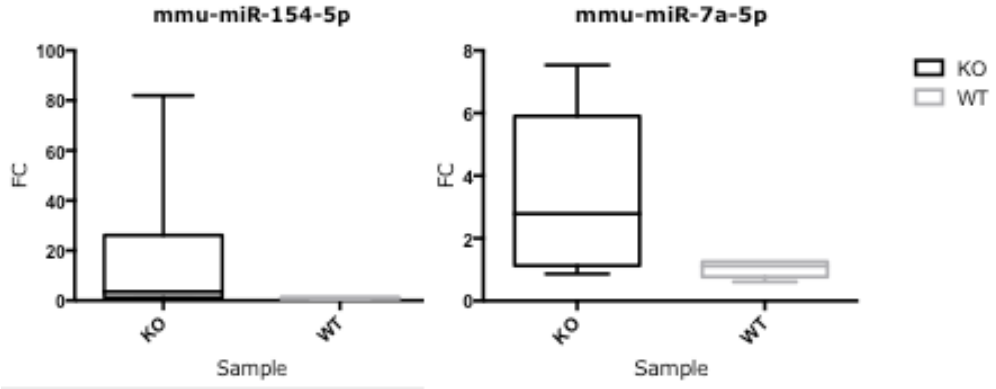


Imagine 22. Upregulated serum miRNA levels in Col6a1-/- 8 months old(n=4) vs. WT (n=5) mice serum but not in Col6a1-/- seven months old col6a1-/-mice serum. Data are presented as a Fold changes (FC) using a log 2 scale (-ΔΔCt). All P-Values are <0.05 (Mann-Whitney U test)

4.6 Common dysregulated miRNAs

The levels of n=23 miRNAs were found to be significantly dysregulated in serum of both the 8-month-old and the 7-month-old KO mice compared to WT animals. Namely, n=2 miRNAs (mmu-miR-7a-5p, mmu-miR-195a-5p) were up-regulated, n=3 were down regulated (mmu-miR-363-3p, mmu-miR-192-3p and mmu-miR-15a-3p) and n=18 were slightly down-regulated (mmu-miR-22-3p, mmu-miR-130b-3p, mmu-miR-192-5p, mmu-miR-362-3p, mmu-miR-19a-3p, mmu-miR-425-3p, mmu-miR-29a-3p, mmu-miR-210-3p, mmu-miR-215-5p, mmu-miR-142-5p, mmu-miR-191-3p, mmu-miR-466g, mmu-miR-130a-3p,

mmu-miR-322-5p, mmu-miR-423-3p, mmu-miR-297a-5p, mmu-miR-19b-3p and mmu-miR-221-3p) in KO serum.



Imagine 23. Common miRNAs upregolated levels in Col6a1-/- mice serum. Data are presented as a Fold changes (FC) using a log 2 scale (-ΔΔCt). All P-Values are <0.05 (Mann-Whitney U test).

4.7 Pathway Analysis

miRNAs are post-transcriptional regulators that act by binding the mRNA and influenced its gene expression. The prediction of the target mRNAs which could be regulated by miRNA changes is highly important.
The hypothesis was that increasing levels of serum miRNAs could exert an inhibitory effect on the target related mRNA.
Therefore, a pathway analysis was carried out using the online tool Diana mirpath v2.0 [37], a software that creates associations between miRNAs and target genes. Once that the target genes has been identified, it could help defining signalling pathways altered in the disease progression.
Hierarchical clustering on both miRNAs and pathways were calculated using squared Euclidean distances in order to highlight miRNA-miRNA and miRNA-pathways relationship obtained from miRBase [38]and Kyoto Encyclopedia of Genes and Genomes (KEGG) [39] respectively.
P-values were calculated using the one-tailed Fisher's exact test and KEGG pathways with p-values <0.05 were considered significant.

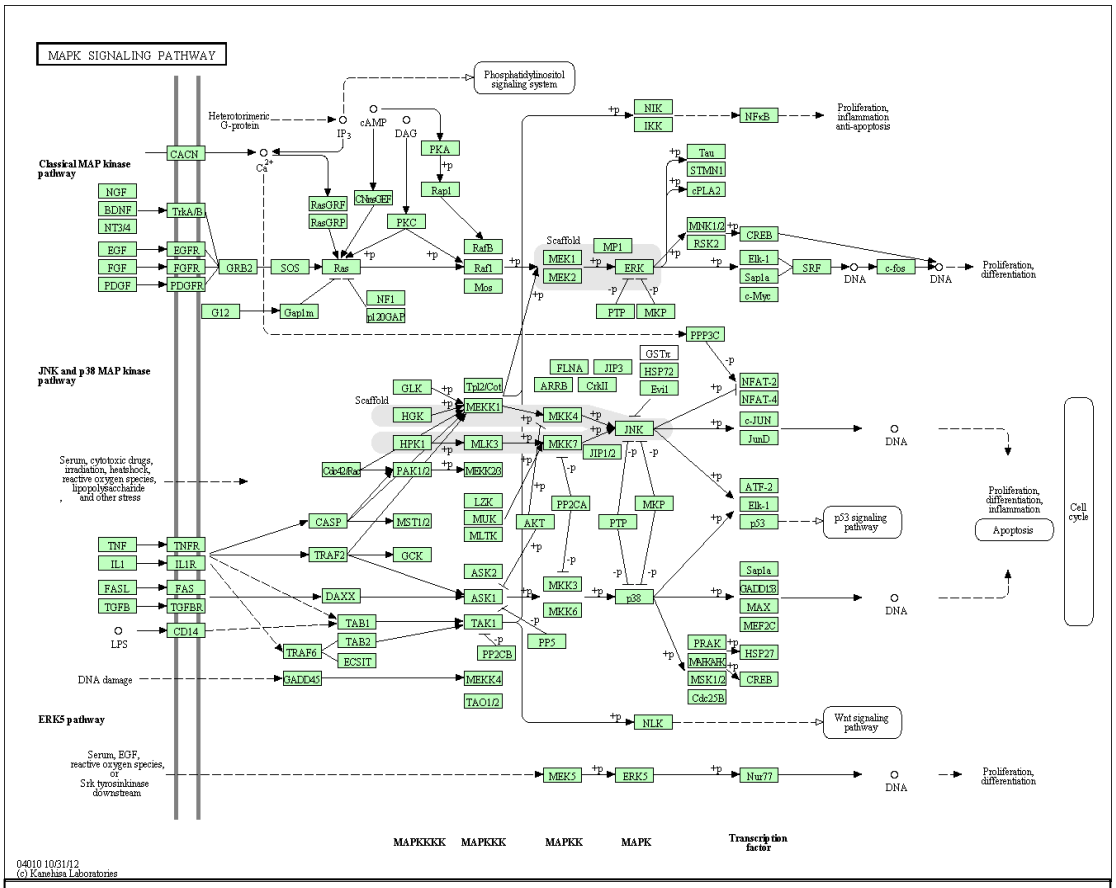
Imagine 24. Up regulated miRNAs in col6a1-/- mice serum vs pathways heat map.Lower significance values are represented by darker coluors. (Map generated from http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index)

Pathways analyses showed that n=4 overexpressed miRNAs in *col6a1*$^{-/-}$ mice serum (mmu-miR-195a-5p, mmu-miR-26a-5p, mmu-let-7c-5p and mmu-let-7b-5p) could potentially regulate genes involved in signalling pathways critical for the development, efficiency and homeostasis of the skeletal muscle.

4.7.1 MAPK signaling pathway

The MAPK signaling pathway regulates an intracellular proteins system that can be activated mainly by hormones, growth factors and environmental stress. Once activated, the MAPK proteins induce phosphorylation of cytoplasmic and nuclear proteins.[40]

The MAPK family consists of four known subfamily-proteins: extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun NH_2 terminal kinase (JNK), p38 and ERK5. These proteins are activated by the phosphorylation of threonine and tyrosine residues by MAP kinase kinases and are inactivated by phosphatases.[41]



Imagine 25. MAPK signalling pathway KEGG (imagine taken from http://www.genome.jp/kegg-bin/show_pathway?mmu04010)

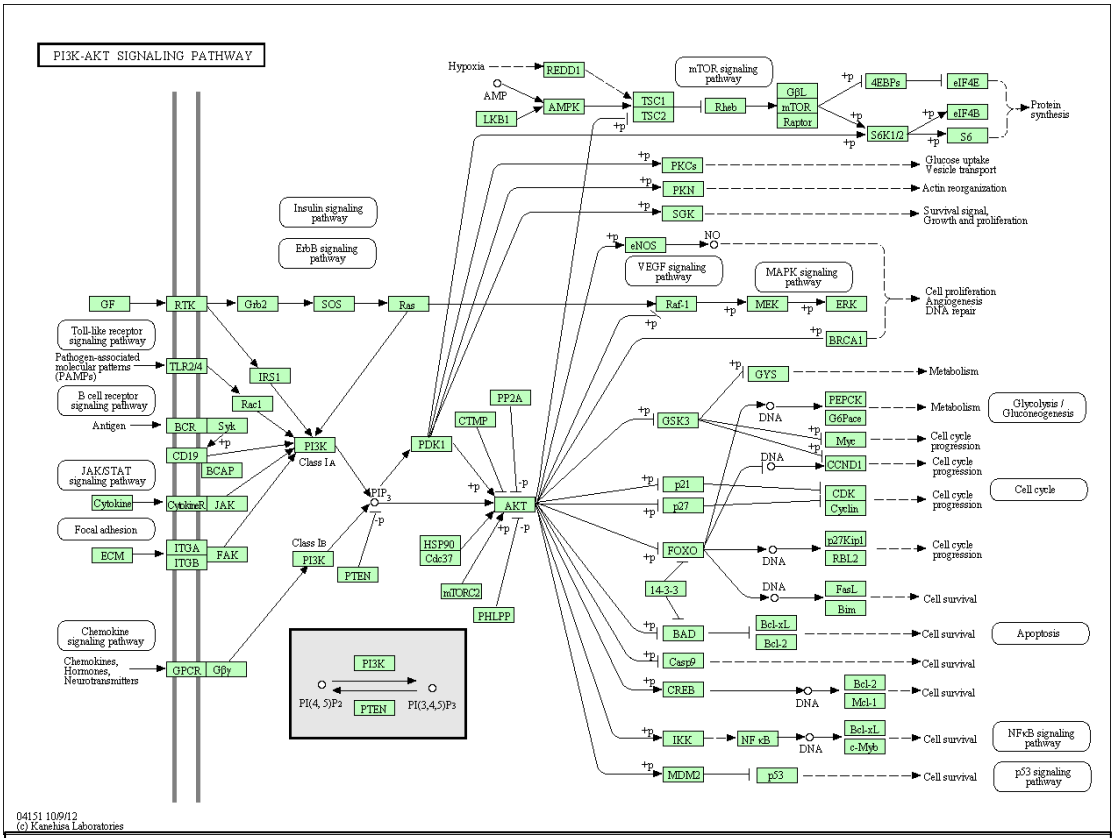
MAPK signaling pathway	
miRNA	**Target gene**
mmu-miR-26a-5p	*Mef2c*
mmu-miR-26a-5p	*Taok1*
mmu-miR-26a-5p	*Map2k4*
mmu-miR-26a-5p	*Mapk9*
mmu-miR-26a-5p	*Sos1*
mmu-miR-26a-5p	*Gng12*
mmu-miR-26a-5p	*Chp1*
mmu-miR-26a-5p	*Ppp3r1*
mmu-miR-26a-5p	*Pla2g4c*
mmu-miR-26a-5p	*Tab2*
mmu-let-7b-5p	*Dusp6*
mmu-let-7b-5p	*Map4k3*
mmu-let-7b-5p	*Nlk*
mmu-let-7b-5p	*Tgfbr1*
mmu-let-7b-5p	*Akt1*
mmu-let-7b-5p	*Braf*
mmu-let-7b-5p	*Nras*
mmu-let-7b-5p	*Crk*
mmu-let-7b-5p	*Flna*
mmu-let-7b-5p	*Ppp3r1*
mmu-let-7b-5p	*Ikbkg*
mmu-let-7b-5p	*Dusp1*
mmu-let-7b-5p	*Mapk1*
mmu-let-7c-5p	*Mapk1*

Imagine 26. Genes belonging to MAPK signalling pathway targeted by mmu-miR-26a-5p, mmu-let-7b-5p and mmu-let-7c-5p

4.7.2 PI3K/Akt signaling pathway

Phosphatidyinositol the 3'-kinase (PI3K) Akt signaling pathway regulates important cellular functions such as transcription, proteins translation, cellular growth and proliferation.

Akt (also known as "protein kinase B," PKB) is the main protein having a key role in this pathway; this protein could be activate by PI3K and could translocate to the plasma membrane where it is finally completely activated by PDK1 and PDK2 kinases.[42]



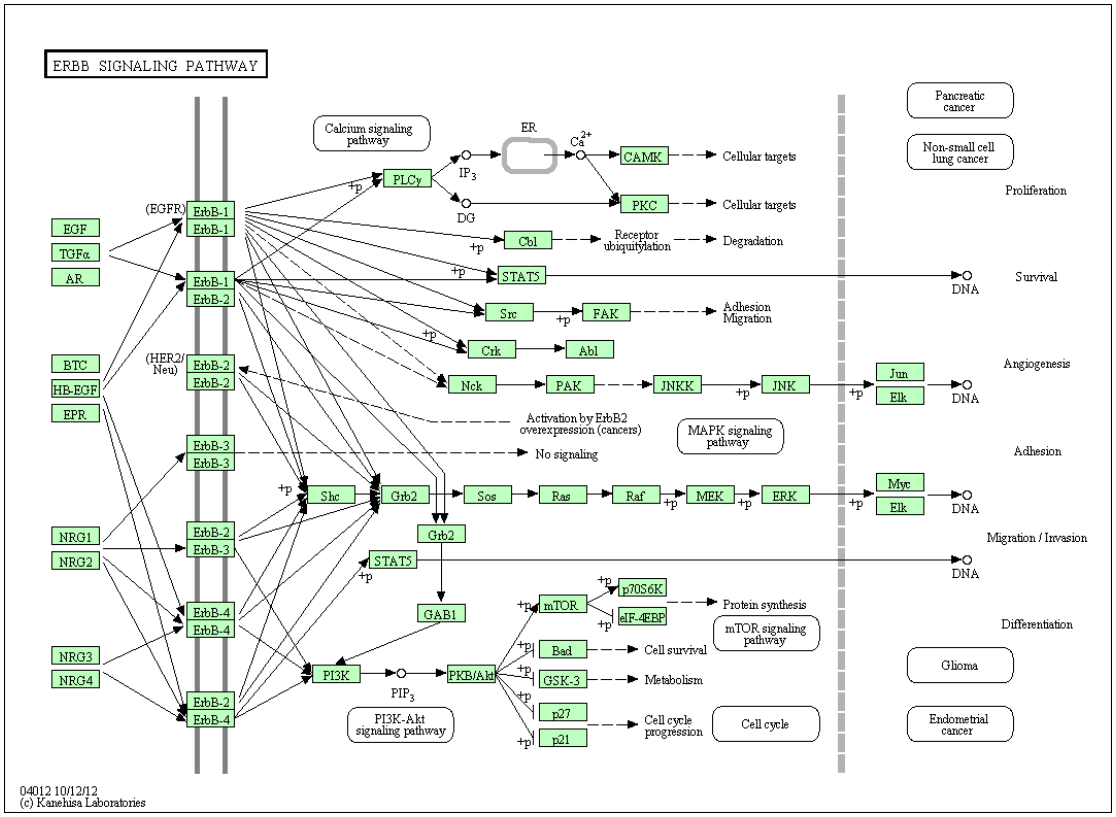
Imagine 27. PI3K/AKT signaling pathway KEGG (imagine taken from http://www.genome.jp/kegg-bin/show_pathway?org_name=mmu&mapno=04151&mapscale=&show_description=hide)

PI3K/Akt signaling pathway	
miRNA	**Target gene**
mmu-miR-195-5p	*Ccnd1*
mmu-miR-195-5p	*Cdk6*

Imagine 28. Genes belonging to PI3K/AKT signaling pathway targeted by mmu-miR-195-5p.

4.7.3 ERBb signaling pathway

The ErbB signaling pathway is composed by four tyrosine kinases receptors: EGFR/ErbB1/Her1, ErbB2/NEU/HER2, ErbB3/HER3 and ErbB4/HER4. All of them present highly homology with erythroblastic leukemia viral oncogene.
This signalling pathway plays an important role in the regulation of cell proliferation, apoptosis and differentiation.



Imagine 24. ErbB signaling pathway KEGG (imagine taken from http://www.genome.jp/kegg-bin/show_pathway?org_name=mmu&mapno=04012&mapscale=&show_description=hide)

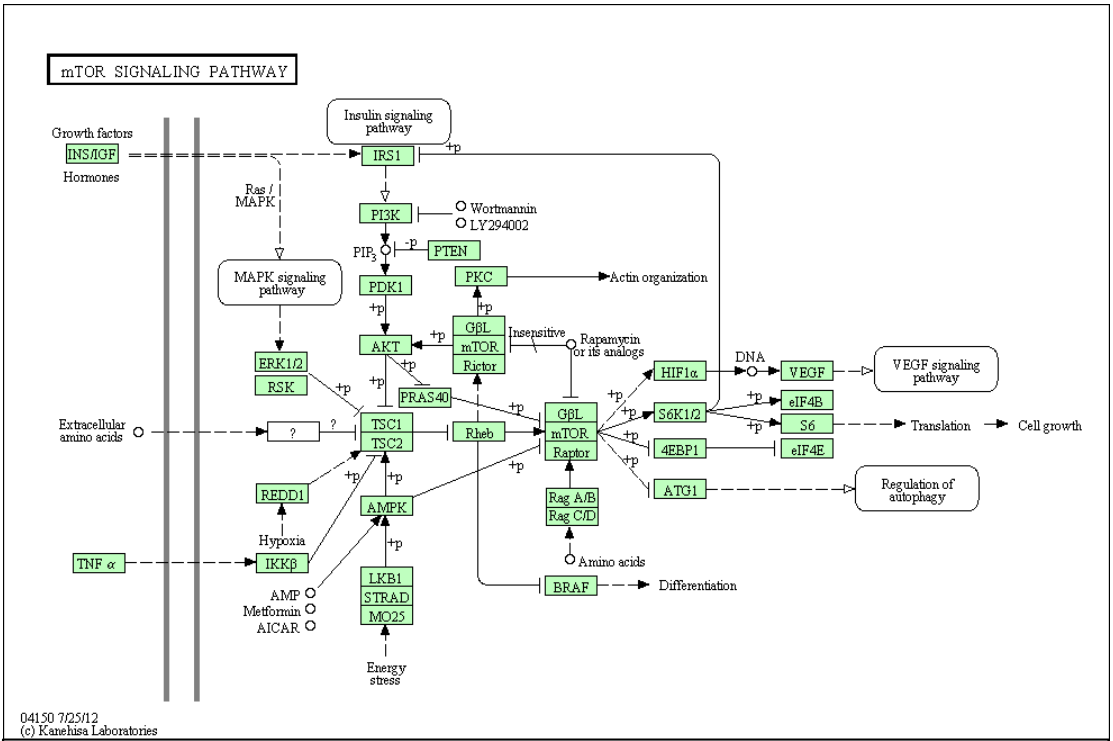
ErbB signaling pathway	
miRNA	**Target gene**
mmu-miR-26a-5p	*Pik3r3*
mmu-miR-26a-5p	*Map2k4*
mmu-miR-26a-5p	*Mapk9*
mmu-miR-26a-5p	*Sos1*
mmu-miR-26a-5p	*Shc4*
mmu-let-7c-5p	*Myc*
mmu-let-7c-5p	*Crk*
mmu-let-7b-5p	*Akt1*
mmu-let-7b-5p	*Braf*
mmu-let-7b-5p	*Nras*
mmu-let-7b-5p	*Crk*

Imagine 29. Genes belonging to ErbB signaling pathway targeted by mmu-miR-26a-5p, mmu-let-7b-5p and mmu-let-7c-5p.

4.7.4 mTOR signaling pathway

The mammalian target of rapamycin (mTOR) acts as regulator of metabolism, growth and cell proliferation.

This pathway is composed by the mTOR protein complex 1 (mTORC1) and mTOR protein complex 2 (mTORC2).[43]



Imagine 30. mTOR signaling pathway KEGG (imagine taken from http://www.genome.jp/kegg-bin/show_pathway?mmu04150)

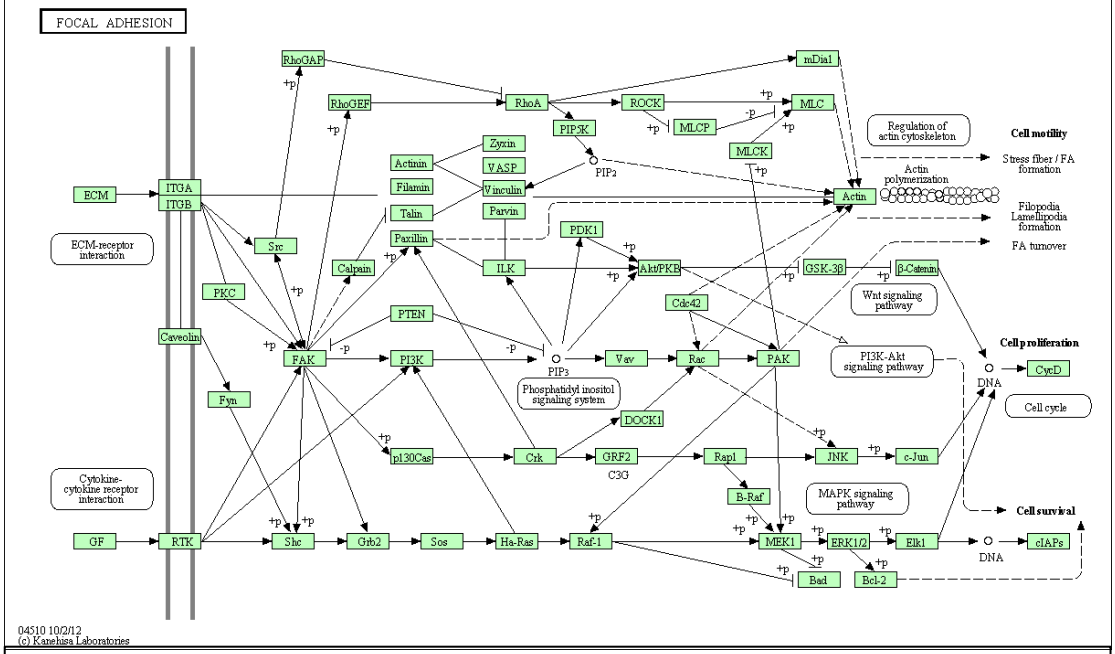
mTOR signaling pathway	
miRNA	**Target gene**
mmu-miR-26a-5p	*Ulk1*
mmu-miR-26a-5p	*Pik3r3*
mmu-miR-26a-5p	*Ddit4*
mmu-miR-26a-5p	*Rragc*

Imagine 31. Genes belonging to mTOR signaling pathway targeted by mmu-miR-26a-5p.

4.7.5 Focal Adhesion signaling pathway

Focal adhesions are structures composed by actin filaments that allow their binding to transmembrane receptors.

The components of focal adhesions link the actin cytoskeleton to the cell membrane and also act as signalling molecules.[44]



Imagine 32. Focal Adhesion signaling pathway KEGG (imagine taken from http://www.genome.jp/kegg/pathway/hsa/hsa04510.html)

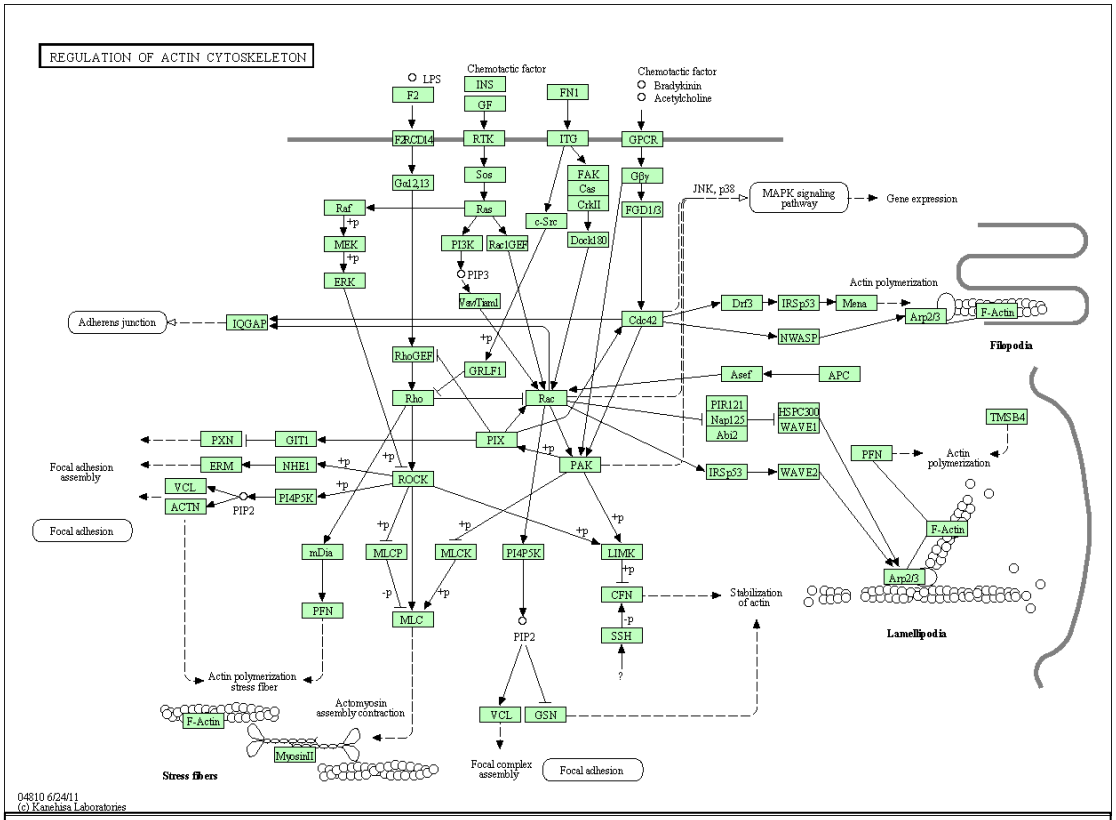
Focal adhesion signaling pathway	
miRNA	**Target gene**
mmu-let-7b-5p	*Vav3*
mmu-let-7b-5p	*Thbs1*
mmu-let-7b-5p	*Akt1*
mmu-let-7b-5p	*Braf*
mmu-let-7b-5p	*Ccnd1*
mmu-let-7b-5p	*Igf1r*
mmu-let-7b-5p	*Rock2*
mmu-let-7b-5p	*Crk*
mmu-let-7b-5p	*Itga4*
mmu-let-7b-5p	*Flna*
mmu-let-7b-5p	*Actg1*
mmu-let-7b-5p	*Col4a2*

Imagine 33. Genes belonging to mTOR signaling pathway targeted by mmu-let-7b-5p.

4.7.6 Actin Cytoskeleton signaling pathway

Actin cytoskeleton is a dense network formed by polymers of actin and other proteins associated with them.

This cellular element is especially important for cell motility and it is responsible for cell shape changings during the cell cycle.[45]



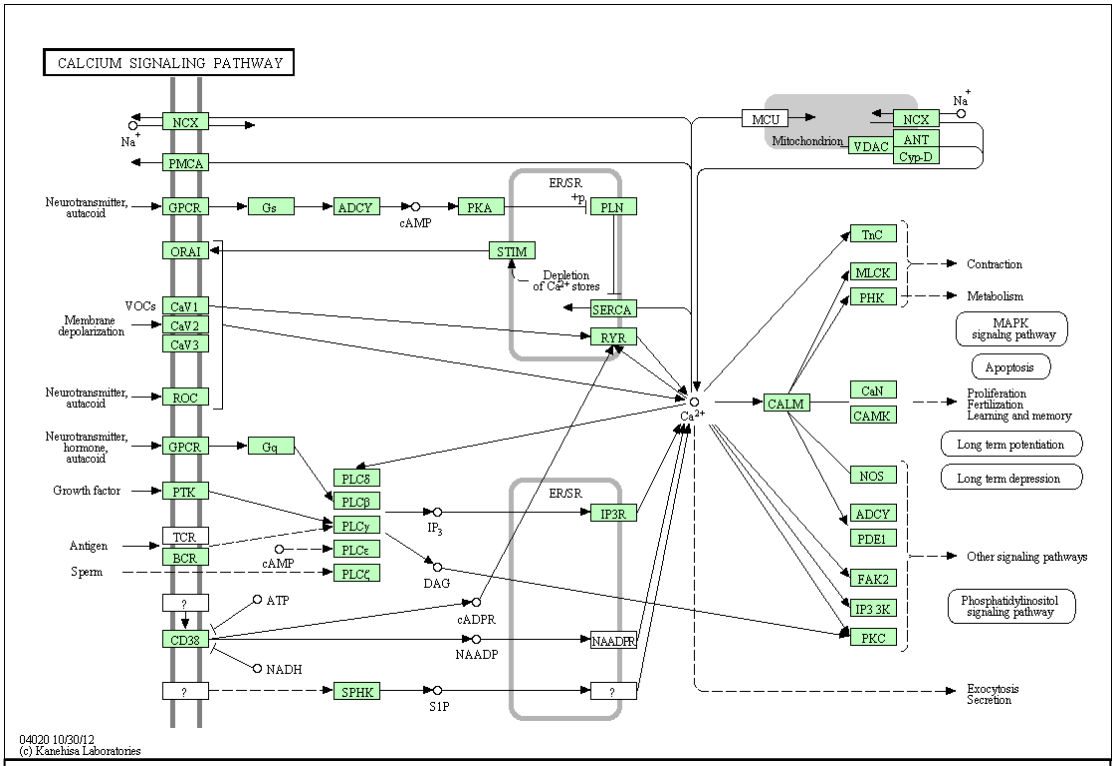
Imagine 34. Actin cytoskeleton signaling pathway KEGG (imagine taken from http://www.genome.jp/kegg-bin/show_pathway?org_name=mmu&mapno=04810&mapscale=&show_description=hide)

Regulation of actin cytoskeleton signaling pathway	
miRNA	**Target gene**
mmu-let-7b-5p	*Vav3*
mmu-let-7b-5p	*Braf*
mmu-let-7b-5p	*Rdx*
mmu-let-7b-5p	*Nras*
mmu-let-7b-5p	*Rock2*
mmu-let-7b-5p	*Crk*
mmu-let-7b-5p	*Itga4*
mmu-let-7b-5p	*Actg1*
mmu-let-7b-5p	*Ssh1*

Imagine 35. Genes belonging to actin cytoskeleton signaling pathway targeted by mmu-let-7b-5p.

4.7.7 Calcium signaling pathway

Ca^{2+} is an essential ion for the proper functioning of skeletal muscle cells, in particular allows the cell to contract and also acts as a second messenger. The concentration of intracellular Ca^{2+} should be perfectly regulated, because the reserves of Ca^{2+} are stored in the endoplasmic reticulum. [46]



Imagine 36. Actin cytoskeleton signaling pathway KEGG (imagine taken from http://www.genome.jp/kegg-bin/show_pathway?org_name=mmu&mapno=04020&mapscale=&show_description=hide)

Calcium signaling pathway	
miRNA	**Target gene**
mmu-let-7b-5p	*Slc25a4*
mmu-let-7b-5p	*Stim1*
mmu-let-7b-5p	*Gnal*
mmu-let-7b-5p	*Atp2a2*
mmu-let-7b-5p	*Atp2b2*
mmu-let-7b-5p	*Slc8a2*
mmu-let-7b-5p	*Ppp3r1*

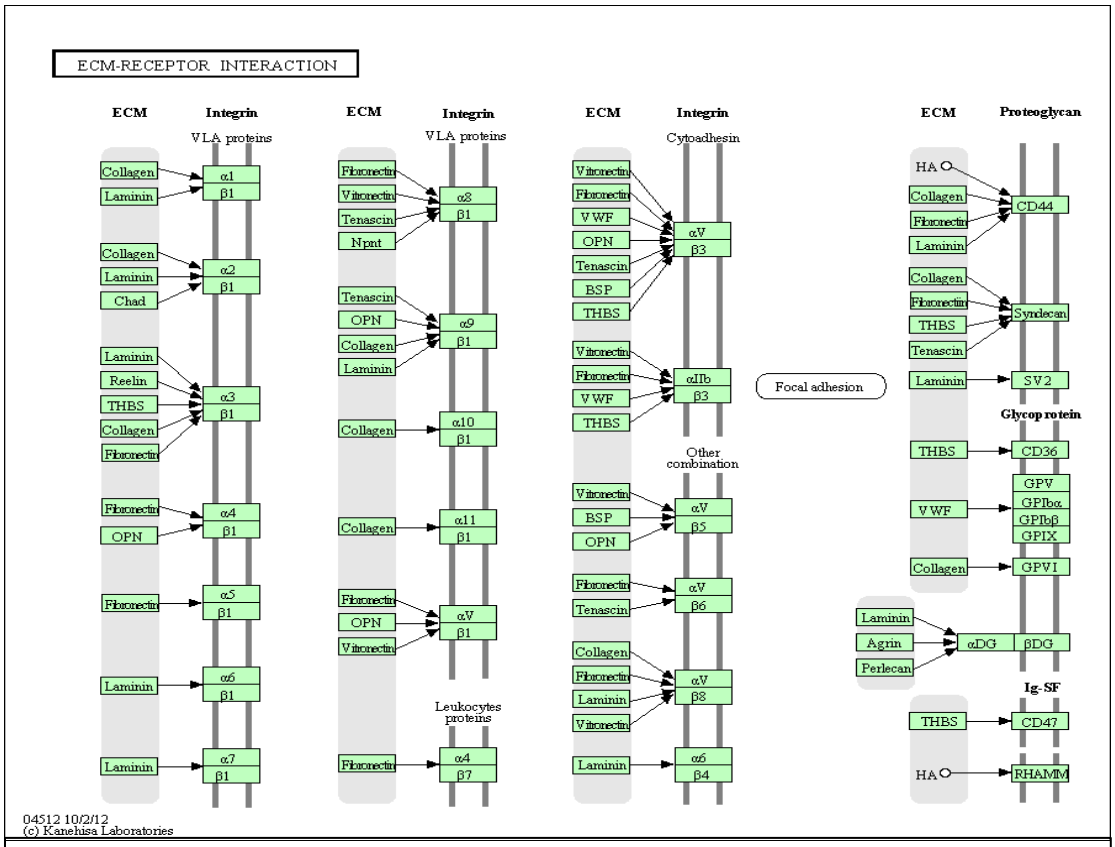
Imagine 37. Genes belonging to Calcium signaling pathway targeted by mmu-let-7b-5p.

4.7.8 ECM-receptors signaling pathway

The extracellular matrix (ECM), mainly composed of glycoproteins, collagen fibrils and proteoglycans, have a structural role and it is also involved in the modulation of many cellular pathways.

Extracellular matrix is in contact with the muscle cell membrane through various proteins, such as integrins.

By these interactions the ECM can act as a regulator of adhesion, differentiation and cell proliferation.[47]



Imagine 38. ECM-receptor signaling pathway KEGG (imagine taken from http://www.genome.jp/kegg-bin/show_pathway?org_name=mmu&mapno=04512&mapscale=&show_description=hide)

ECM-receptor signaling pathway	
miRNA	**Target gene**
mmu-miR-26a-5p	*Itgb1*

Imagine 39. Genes belonging to ECM-receptor signaling pathway targeted by mmu-miR-26a-5p.

5.Discussion

Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) are a subtype of congenital muscular dystrophies (CMDs) caused by mutations in the genes coding for the collagen VI main chains (*COL6A1, COL6A2* and *COL6A3*). Collagen VI is a ubiquitous extracellular matrix (ECM) protein synthesized by fibroblasts and skeletal muscle cells having both structural and signalling functions [10].

By the interaction with other ECM proteins such as integrins, decorin, biglican, fibronectin and basal lamina type IV collagen [21, 22], collagen VI represents a bridge between muscle cells and surrounding ECM, playing an important signalling role [1].

UCMD and BM diagnosis requires muscle biopsy, which is an invasive test that allows to study the fibers morphology and

allows the identification of the characteristic histological features observed in collagenopaties [4].

The purpose of this project was to study the serum expression patterns of a large group of miRNAs by quantitative Real-Time polymerase chain reaction (qPCR) in collagen VI−deficient mice (*col6a1*$^{-/-}$*)* [34] in order to find dysregulated miRNAs which could be useful in future as no invasive biomarker for the diagnosis and the follow-up of UCMD and BM patients.

Col6a1$^{-/-}$ mouse is considered the ideal animal model to study the collagen VI-related myopathy since muscle histophatology shows marked signs of myopathy like muscle necrosis and regenerating fibers [34].

MiRNAs are non-coding RNAs (ncRNAs) long 22 nucleotides that act as post transcriptional inhibitors binding RNA targets and inhibiting their expression; these oligonucleotides are easily detectable in different body tissues.[30]

Recent studies showed that miR-1/206 and miR-133a/ 133b family members are specifically expressed in skeletal and cardiac muscle.[31]

This project identified 333 miRNA expressed in *col6a1*$^{-/-}$ mice serum and showed that 6,5% of them were significantly differentially expressed between *Col6a1*$^{-/-}$ and WT mice serum.

A total of 51 miRNAs were significantly dysregulated; 17/51 miRNAs resulted up-regulated and 34/51 down-regulated.

Pathway analyses coupled to research literature findings were performed in order to identify the most significant dysregulated miRNAs which could be used as candidates for further proof-analysis in human serum.
The performed pathway analysis were based on overexpressed miRNA because our hypothesis was that the characteristic inhibitory effect of each miRNA may increase in relation to its serum amount, however literature research was used also to find correlation between down-regulated miRNAs and other CMDs forms.
Pathway analysis associates miRNA to the relatives target genes and are based on previous studies or made by a specific algorithm that create miRNA-target associations (analysing the miRNA-target sequence homology) and including the target genes involvement in a specific signalling-pathway.
Interestingly, we have identified 4 significantly up-regulated miRNAs in *col6a1*$^{-/-}$ mice serum (mmu-miR-195a-5p, mmu-miR-26a-5p, mmu-let-7c-5p and mmu-let-7b-5p) that potentially could bind transcripts of genes involved in crucial pathway for skeletal muscle cells development, differentiation or coding for ECM proteins.
Mmu-let-7c-5p and mmu-let-7b-5p bind mitogen-activated protein kinase 1 (*Mapk1)* and mmu-let-7b-5p bind mitogen binds Mitogen-Activated Protein Kinase 4 (*Map4k3)* gene, belonging to the MAPK signaling pathway which is activated in human skeletal muscle during exercise. [40]
Grendinger and colleagues found that MAPK signalling pathway activation controls the C2 muscle cell lines differentiation increasing Myo-D family proteins (Myf5 and Myo-D) levels [48] while Burks and collaborators suggested that MAPKs (a Transforming growth factor beta TGF-β protein members) plays an important role in skeletal muscle pathogenesis and they are involved in several inherited neuromuscular disorders [49].
Moreover mmu-let-7c-5p and mmu-let-7b-5p bind the insulin-like growth factor 1 gene (*Igf1*) a PIK3/Akt pathway activator.
Glass and colleagues suggested that activation of PIK3/Akt pathway results in myostatin inhibition and atrophy mediators MuRF1 and MAFbx (atrogin1) up-regulation in skeletal muscle [50].
Previous studies showed that IGF-1 expression lead to muscle mass and strength increase by promoting the regeneration [51-55] and that IGF-1 reduced fibrosis in *mdx mice*. [56]

In this context, an overexpression of both, mmu-let-7c-5p and mmu-let-7b-5p could reduce the skeletal muscle cells differentiation acting to MAPK signaling pathway and exert inhibitory effect reducing the IGF-1 activity in *col6a1*$^{-/-}$ mice.
An excess of mmu-let-7b-5p may also exert adverse effects on the muscle contraction linking the Stromal Interaction Molecule 1
gene (*Stim1*) a gene encoding for a endoplasmic reticulum calcium receptor whose malfunction caused by mutations was associated to aggregates tubular myopathy form.[46]
In addition to this effect, mmu-let-7b-5p could modify the ECM composition and cytoskeleton organization by the interaction with several proteins belonging to these structures.
Among the potential target genes of mmu-miR-26a-5p I have identified as interested candidate the integrin *β1* (*Itgβ1)* gene.
It has been shown that *β1* integrin is important for NMJ (neuromuscular junction) development in skeletal muscle and lack of this receptor is linked to an interruption of the connection between skeletal muscle and motor neurons. [57]
β1 integrin are also involved in signalling connecting skeletal muscle to the surrounding ECM. [58]
Pathway analysis highlighted a link between mmu-miR-26a-5p and several mTOR signalling pathway genes which often lower expression levels associated with myopathy conditions.
Interestingly, Risson and colleagues found that mTOR^{-} mouse had lower levels of dystrophin and related Dystrophin-Glycoprotein Complex (DGCs); this observations have been linked to uthrophin overexpression as observed in mdx mice.[43]
The mmu-miR-26a-5p could have then negative effects on the NMJ formations and promoting the dystrophic phenotype development and progression by mTOR signalling alterations.
This work has also shown that mmu-miR-29b-3p and mmu-miR-29a-3p were significantly down-regulated in of *Col6a1*$^{-/-}$ mice serum.
Wang and colleagues found that in myoblast cells loss of miR-29 contributes to dystrophic changes in DMD muscles and its down regulation has been identified in *mdx* mice serum.[59]
This study clearly indicated that several miRNAs are significantly dysregulated in *Col6a1*$^{-/-}$ mice serum compared to WT and among them mmu-miR-195a-5p,

mmu-miR-26a-5p, mmu-let-7c-5p and mmu-let-7b-5p could potentially bind transcripts of genes involved in important signalling pathways for development and differentiation of skeletal muscle cells.
Their serum upregulation may have effects on the protein regulation resulting in a molecular pathways alteration with the consequent histological changes observed in muscles of collagen VI−deficient mice.
Nevertheless, further functional studies using miRNA mimics and inhibitors in cell cultures from the same *Col6a1*$^{-/-}$ mice are essential in order to confirm the above described hypothesis.
A study of *Col6a1*$^{-/-}$ mice muscle miRNA levels will also be useful to verify if candidate miRNA expression patterns are different between serum and muscle of *Col6a1*$^{-/-}$ mice, as demonstrated in *mdx* mouse by Roberts and colleagues.[60]
Finally, the results obtained in this study have to been considered preliminary data and they could be potentially used in order to select a miRNA panel that could be tested on serum of affected patients.
Serum levels of the dysregulated miRNAs identified in this study (upregulated ;mmu-miR-195a-5p, mmu-miR-26a-5p, mmu-let-7c-5p and mmu-let-7b-5p and downregulated:mmu-miR-29b-3p and mmu-miR-29a-3p) will be analysed performing qPCR in a large cohort of BM and UCMD patients.
If a panel of dysregulated miRNAs will be identified, this will be an extremely powerful tool that may allow to skip the painful and invasive muscle biopsy procedure.

6.Bibliography

1. **Bernardi, P. and P. Bonaldo,** *Mitochondrial dysfunction and defective autophagy in the pathogenesis of collagen VI muscular dystrophies.* **Cold Spring Harb Perspect Biol, 2013. 5(5): p. a011387.**

2. **Allamand, V., et al.,** *ColVI myopathies: where do we stand, where do we go?* **Skelet Muscle, 2011. 1: p. 30.**

3. **Emery, A.E.,** *The muscular dystrophies.* **Lancet, 2002. 359(9307): p. 687-95.**

4. **Mercuri, E. and F. Muntoni,** *Muscular dystrophies.* **Lancet, 2013. 381(9869): p. 845-60.**

5. Dalkilic, I. and L.M. Kunkel, *Muscular dystrophies: genes to pathogenesis.* **Curr Opin Genet Dev, 2003. 13(3): p. 231-8.**

6. Guglieri, M., et al., *Limb-girdle muscular dystrophies.* **Curr Opin Neurol, 2008. 21(5): p. 576-84.**

7. Bushby, K., et al., *Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care.* **Lancet Neurol, 2010. 9(2): p. 177-89.**

8. Rowland, L.P., T.A. Pedley, and H.H. Merritt, *Merritt's neurology.* ***12th ed2010*****, Philadelphia, PA: Lippincott Williams & Wilkins. xxi, 1172 p.**

9. Lynn, D.J., H.B. Newton, and A. Rae-Grant, *The 5-minute neurology consult.* **2nd ed2012, Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health. p.**

10. Buonocore, G., R. Bracci, and A.M. Weindling*, Neonatology : a practical approach to neonatal diseases2010***, Milan ; New York: Springer. 1348 p.**

11. Mercuri, E. and F. Muntoni, *The ever-expanding spectrum of congenital muscular dystrophies.* **Ann Neurol, 2012. 72(1): p. 9-17.**

12. Becker, A.K., et al., *A Structure of a Collagen VI VWA Domain Displays N and C Termini at Opposite Sides of the Protein. Structure,* **2014. 22(2): p. 199-208.**

13. Bonaldo, P., et al., *Alpha 1 chain of chick type VI collagen. The complete cDNA sequence reveals a hybrid molecule made of one short collagen and three von Willebrand factor type A-like domains.* **J Biol Chem, 1989. 264(10): p. 5575-80.**

14. Doliana, R., P. Bonaldo, and A. Colombatti, *Multiple forms of chicken alpha 3(VI) collagen chain generated by alternative splicing in type A repeated domains.* **J Cell Biol, 1990. 111(5 Pt 1): p. 2197-205.**

15. Fitzgerald, J., et al., *Three novel collagen VI chains, alpha4(VI), alpha5(VI), and alpha6(VI).* **J Biol Chem, 2008. 283(29): p. 20170-80.**

16. Gara, S.K., et al., *Three novel collagen VI chains with high homology to the alpha3 chain.* **J Biol Chem, 2008. 283(16): p. 10658-70.**

17. Whittaker, C.A. and R.O. Hynes, *Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere.* **Mol Biol Cell, 2002. 13(10): p. 3369-87.**

18. **Rossmann, M.G., D. Moras, and K.W. Olsen,** *Chemical and biological evolution of nucleotide-binding protein.* **Nature, 1974. 250(463): p. 194-9.**

19. **Lee, J.O., et al.,** *Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18).* **Cell, 1995. 80(4): p. 631-8**

20. **Ricard-Blum, S., B. Dublet, and M. Van der Rest,** *Unconventional collagens : types VI, VII, VIII, IX, X, XII, XIV, XVI, and XIX.* **Protein profile2000, Oxford ; New York: Oxford University Press. xv, 155 p.**

21. **Pfaff, M., et al.,** *Integrin and Arg-Gly-Asp dependence of cell adhesion to the native and unfolded triple helix of collagen type VI.* **Exp Cell Res, 1993. 206(1): p. 167-76.**

22. **Burg, M.A., et al.,** *Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules.* **J Biol Chem, 1996. 271(42): p. 26110-6.**

23. **Hill, J.A. and E.N. Olson,** *Muscle : fundamental biology and mechanisms of disease.* **1st ed2012, London ; Waltham, MA: Academic Press.**

24. **Merlini, L., et al.,** *Autosomal recessive myosclerosis myopathy is a collagen VI disorder.* **Neurology, 2008. 71(16): p. 1245-53.**

25. **Dubowitz, V., C.A. Sewry, and A. Oldfors,** *Muscle biopsy : a practical approach: Expert Consult 2013,* **Philadelphia: Saunders/Elsevier. 592.**

26. **Merlini, L. and P. Bernardi,** *Therapy of collagen VI-related myopathies (Bethlem and Ullrich).* **Neurotherapeutics, 2008. 5(4): p. 613-8.**

27. **Pritchard, C.C., H.H. Cheng, and M. Tewari,** *MicroRNA profiling: approaches and considerations.* **Nat Rev Genet, 2012. 13(5): p. 358-69.**

28. **Hrdlickova, B., et al.,** *Genetic variation in the non-coding genome: Involvement of micro-RNAs and long non-coding RNAs in disease.* **Biochim Biophys Acta, 2014.**

29. **Bibekanand Mallick, Zhumur Ghosh,** *Regulatory RNAS.* **2012, New York: Springer.**

30. **Eisenberg, I., et al.,** *Distinctive patterns of microRNA expression in primary muscular disorders.* **Proc Natl Acad Sci U S A, 2007. 104(43): p. 17016-21.**

31. **Williams, A.H., et al.,** *MicroRNA control of muscle development and disease.* **Curr Opin Cell Biol, 2009. 21(3): p. 461-9.**

32. Cacchiarelli, D., et al., *miRNAs as serum biomarkers for Duchenne muscular dystrophy.* **EMBO Mol Med, 2011. 3(5): p. 258-65.**

33. Zaharieva, I.T., et al., *Dystromirs as serum biomarkers for monitoring the disease severity in Duchenne muscular Dystrophy.* **PLoS One, 2013. 8(11): p. e80263.**

34. Bonaldo, P., et al., *Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy.* **Hum Mol Genet, 1998. 7(13): p. 2135-40.**

35. Braasch, D.A., Y. Liu and D.R. Corey, *Antisense inhibition of gene expresssion in cells by oligonucleotides incorporating locked nucleic acids: effect of mRNA target sequence and chimera design.* **Nucleic Acids Res, 2002. 30(23): p.5160-7.**

36. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* **Proc Natl Acad Sci U S A, 1998. 95(25): p. 14863-8.**

37. Vlachos, I.S., et al., *DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways.* **Nucleic Acids Res, 2012. 40(Web Server issue): p. W498-504.**

38. Kozomara, A. and S. Griffiths-Jones, *miRBase: integrating microRNA annotation and deep-sequencing data.* **Nucleic Acids Res, 2011. 39(Database issue): p. D152-7.**

39. Kanehisa, M., et al., *KEGG for integration and interpretation of large-scale molecular data sets.* **Nucleic Acids Res, 2012. 40(Database issue): p. D109-14.**

40. Aronson, D., et al., *Exercise stimulates the mitogen-activated protein kinase pathway in human skeletal muscle.* **J Clin Invest, 1997. 99(6): p. 1251-7.**

41. Shi, H., et al., *Modulation of skeletal muscle fiber type by mitogen-activated protein kinase signaling.* **FASEB J, 2008. 22(8): p. 2990-3000.**

42. Osaki, M., M. Oshimura, and H. Ito, *PI3K-Akt pathway: its functions and alterations in human cancer.* **Apoptosis, 2004. 9(6): p. 667-76.**

43. Risson, V., et al., *Muscle inactivation of mTOR causes metabolic and dystrophin defects leading to severe myopathy.* **J Cell Biol, 2009. 187(6): p. 859-74.**

44. Goetsch, K.P., et al., *ROCK-2 is associated with focal adhesion maturation during myoblast migration.* **J Cell Biochem, 2014. 115(7): p. 1299-307.**

45. **Schmidt, A. and M.N. Hall,** *Signaling to the actin cytoskeleton.* **Annu Rev Cell Dev Biol, 1998. 14: p. 305-38.**

46. **Bohm, J., et al.,** *Constitutive activation of the calcium sensor STIM1 causes tubular-aggregate myopathy.* **Am J Hum Genet, 2013. 92(2): p. 271-8.**

47. **Choquet, D., D.P. Felsenfeld, and M.P. Sheetz,** *Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages.* **Cell, 1997. 88(1): p. 39-48.**

48. **Gredinger, E., et al.,** *Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells.* **J Biol Chem, 1998. 273(17): p. 10436-44.**

49. **Burks, T.N. and R.D. Cohn,** *Role of TGF-beta signaling in inherited and acquired myopathies.* **Skelet Muscle, 2011. 1(1): p. 19.**

50. **Glass, D.J.,** *PI3 kinase regulation of skeletal muscle hypertrophy and atrophy.* **Curr Top Microbiol Immunol, 2010. 346: p. 267-78.**

51. **Barton-Davis, E.R., et al.,** *Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function.* **Proc Natl Acad Sci U S A, 1998. 95(26): p. 15603-7.**

52. **Chakravarthy, M.V., et al.,** *Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway.* **J Biol Chem, 2000. 275(46): p. 35942-52.**

53. **Lawlor, M.A. and P. Rotwein,** *Insulin-like growth factor-mediated muscle cell survival: central roles for Akt and cyclin-dependent kinase inhibitor p21.* **Mol Cell Biol, 2000. 20(23): p. 8983-95.**

54. **Lynch, G.S., et al.,** *IGF-I treatment improves the functional properties of fast- and slow-twitch skeletal muscles from dystrophic mice.* **Neuromuscul Disord, 2001. 11(3): p. 260-8.**

55. **Musaro, A., et al.,** *Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle.* **Nat Genet, 2001. 27(2): p. 195-200.**

56. **Barton, E.R., et al.,** *Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice.* **J Cell Biol, 2002. 157(1): p. 137-48.**

57. Schwander, M., et al., *Beta1 integrins in muscle, but not in motor neurons, are required for skeletal muscle innervation.* **J Neurosci, 2004. 24(37): p. 8181-91.**

58. Belkin, A.M., et al., *Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells.* **J Cell Biol, 1996. 132(1-2): p. 211-26.**

59. Wang, L., et al., *Loss of miR-29 in myoblasts contributes to dystrophic muscle pathogenesis.* **Mol Ther, 2012. 20(6): p. 1222-33.**

60. Roberts, T.C., et al., *Expression analysis in multiple muscle groups and serum reveals complexity in the microRNA transcriptome of the mdx mouse with implications for therapy.* **Mol Ther Nucleic Acids, 2012. 1: p. e39.**

61. Mestdagh, P., et al., *A novel and universal method for microRNA RT-qPCR data normalization.* **Genome Biol, 2009. 10(6): p. R64.**

7.Acknowledgements

Firstly, I am deeply indebted to my supervisor Professor Elena Pegoraro who introduced me to the "research-world", and for her guidance and support throughout my time at the University of Padova.

I would like to express my gratitude to Professor Muntoni who gave me the opportunity to have a research experience in London and develop this project at University College London.

I would like to thank Irina Zaharieva for the assistance and technical guidance provided throughout the project.

I would like to thank my family: my parents Carla and Pietro, my brothers Tommaso and Enrico, and my grandparents Tommaso, Carlo, Maria and Emma.They are the most important people in my life, who support me with their love every day. This thesis is dedicated to them.

Finally, a big thank you to my friends and colleagues of University of Padova and University College London, and to F.C. Internazionale Milano for keeping me sane over the past few months.