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TRANSCRIPTIONAL PROFILING IN DUCHENNE MUSCULAR DYSTROPHY REVEALED A PROFOUND DEREGULATION OF BOTH CIRCADIAN RHYTHM GENES AND GENES INVOLVED IN MUSCLE REGENERATIVE PROCESS AS POSSIBLE NEW DISEASE BIOMARKERS

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1. INTRODUCTION

1.1. Dystrophinopaties: general overview

Dystrophin gene (DMD), located in Xp21, encodes for dystrophin protein and spans 2,2 Mb representing the largest gene, accounting for 1% of the entire human genome (Lander et al. 2001). It comprises 78 constitutive exons and 7 isoform-specific exons (Purkinje, Muscle, Brain, Dp116, DP140, Dp260, Dp71) with just as many introns, some of them being of huge dimensions, containing promoters for the 7 dystrophin isoforms.

Such large introns represent a great expense in terms of energy for gene transcription and splicing. In addition the richness in repetitive elements (like LINE1, and SINEs) might be related to dystrophin gene high mutation rate (currently more than 50% of DMD mutations occurs the novo). These large regions make indeed more prone the gene to broad recombining events via NAHR or NHEJ (Ferlini et al. 2013) that cause rearrangements.

The dystrophin protein is a "rod-shaped" protein of 427kDa in molecular weight, composed of four domains: 1) N-terminal domain, that mediates alpha-actin interaction promoting the anchoring with sarcomere contractile apparatus; 2) the central rod-domain that comprises 24 spectrin-like repeats which are interrupted by 4 proline rich hinge-regions; these regions contribute to protein flexibility via their force transducing functions during sarcomere contraction. Repeats 16 and 17 are essential for the anchoring of neuronal nitric oxide synthase (nNOS), a key element in muscle blood flux regulation and dystrophin-mediated signal pathways, this last mediated by alpha-syntrophin binding site at the 3' of the gene (exon 73-75) (Ervasti et al. 1993). In dystrophinopathies, protein absence leads to a down regulation of this enzyme possibly mediating the damage persistence (exercise induced paradoxic vasoconstriction) (Arechevala-Gomeza et al. 2012); 3) cysteine-rich domain contains 15 cysteine residues and mediates dystrophin anchoring to sarcolemma by means of β-dystroglican; 4) C-terminal domain mediates the interaction with α and β syntrophins (Ahn et al. 1995). Syntrophins are part of the dystrophin-associated glycoproteins complex (DAG), made by α and β dystroglican, the sarcoglycan-sarcospan complex and the cytoplasmic complex (which comprise dystrophin, α-dystrobrevin and α-syntrophin). DAG mediates the anchoring to laminin-α2, exerting the structural function of mechanic-transducer between muscle fibers and extracellular matrix, and thus maintaining muscle integrity. In particular, dystrophin is expressed in costamers, subsarcolemma, in cardiac and skeletal (striated) muscles, post-synaptic membrane of neuromuscular junction and post-synaptic neuronal regions, including

Purkinje cells in the cerebellum, amigdala, limbic lobe and frontal cortex (Cullen et al. 1990; Lidov et al., 1990; Sealock et al., 1991; Hendriksen et al. 2016, Petkova et al. 2016). Dystrophin is also expressed in heart T-tubules where it has a specific function in excitation-contraction coupling (Muntoni 2003). A part from the structural properties, its role is well known in extracellular signaling, modulating some metabolic, growth and regenerative muscle functions (Rando et al., 2001, Church et al. 2014; Acuna et al. 2014).

As mentioned above, the dystrophin gene is highly prone to spontaneous mutations because of its huge introns: indeed roughly 50% of mutations are *de novo* and this may account for the relatively high prevalence (1 in 5000 male birth) of dystrophinopathies in the population. Mutations that occur more frequently are large genomic rearrangements as deletions and duplications, that represent together up to 75% of identifiable alterations (65% deletion, 10% duplication) (Ferlini et al. 2013). They recognize their breakpoints mainly in introns via non-homologous recombination with two main hotspots, located in introns 3-7 and 44 (Suzuki et al. 2016; Winnard et al. 1995; Nakamura et al. 2016).

Phenotypic consequences of deletions are due to the Monaco frame theory (Monaco et al. 1998). In general (and not only for the dystrophin gene) mutations, any type, which disrupt the reading frame cause a premature truncated protein and therefore a complete absence of the protein product, while mutations, even large deletions, maintaining the reading frame (for dystrophin is "3") allow the production of a shorter but partially functional protein, therefore causing a milder phenotype (Becker muscular dystrophy or BMD). In the case of duplications, the frame theory is not always respected, since other mechanisms (alternative splicing, novel ATG starting site) may complicate the genotype-phenotype correlation.

The "frame rule" is the base of the irrelevancy of mutation dimensions: literature reports cases of large deletions/duplications removing/adding even more than 10 exons associated with BMD phenotype, the reasons residing in the rule that keeping both N- and C-terminal domains resulted in a "mini" dystrophin still maintaining their anchoring properties to the extracellular matrix (Angelini et al. 1990) and thus safeguarding the mechanotransducer function (Figure 1).

The remaining 25% of the mutations are small nucleotide changes, as missense, nonsense, frameshifting, small indels, small duplications and inversions, and splicing site mutations (Leiden DMD pages, http://www.dmd.nl). Nonsense and frameshifting mutations generally lead to a DMD phenotype. Missense mutations causing an amino acid substitution in the

final protein sequence are associated with both DMD and BMD, and their pathogenic role might be related to domain functions, exon skipping events or isoform production (Aartsma Rus et al. 2016; Finkel 2010).

Roughly 7% of the small mutations are splicing mutations, either affecting the canonical splice sites, or exonic splicing enhancers (ESEs), that regulate the exon incorporation into the mature transcript. Phenotypic effect of canonical splice site mutations is invariably DMD or severe phenotype due to an alteration in the correct splicing mechanism and final omission or incorporation of the entire or part of introns or exons leading to a frame-shift and truncated protein production.

Other splicing mutations should be often evaluated by transcript studies in order to get their phenotypic consequences (Tuffery-Giraud et al. 2017).

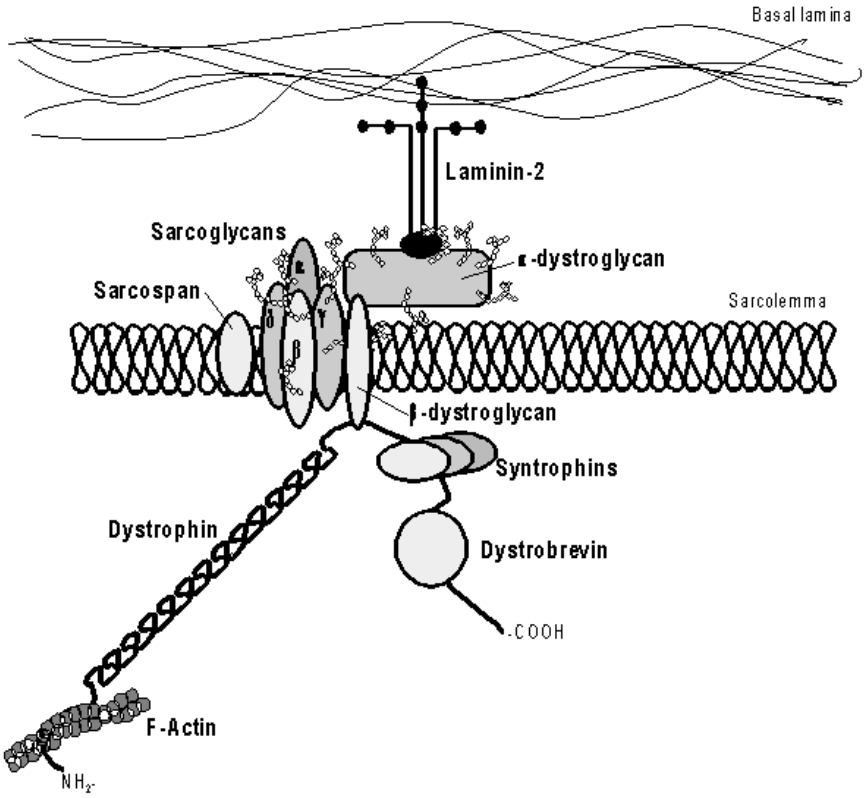

Figure 1 - Dystrophin subsarcolemmatic localization and its interaction with DAG proteins (from Leiden Muscular dystrophy pages, www.dmd.nl/DGC).

Dystrophinopaties are X-linked recessive diseases: hemizygous males for the mutation are affected, whereas heterozygous female are carriers, 99% asymptomatic or very rarely with different degrees of clinical involvement (Stark 2015).

Female carriers have a 50% probability of transmitting the mutated allele to offspring regardless of gender: daughters will be carriers, whereas sons will be affected, or healthy if receiving the wild type allele.

The pathophysiological onset of the disease directly links to the quali/quantitative defect of the dystrophin protein: in case of dystrophin absence, or severe reduction, muscle fibers are more prone to membrane damage due to muscle contraction. The resulting leaky membrane leads to the onset of a calcium-mediated degenerative process that culminates in inflammation and consequent muscle fibers necrosis (De Luca et al. 2002). Dystrophin absence also produces the nitric oxide cytosolic delocalization: as a consequence, the impairment to counterbalance contraction-induced vasoconstriction leads to functional ischemia and finally a worsening of muscle damage (Constantin 2014).

The combination of all these factors, that act at the same time in damaged muscle, induce an oxidative stress due to the release of reactive oxygen species (ROS), which further worsen muscle degeneration. At the beginning of this process there is an attempt to react via regenerative processes (myoblasts recruitment and differentiation); however, after a number of degenerative-regenerative cycles there is a failure with the complete fibrotic substitution of muscle tissue, that is mainly induced by the activation of Tgfb1 pathway. Various dystrophic hallmarks can be seen in histological samples: fiber type disproportion, muscle necrosis and white blood cell infiltrates, myoblast recruitment and proliferation, fibrosis.

Dystrophinopathies diagnosis can be made at three levels: clinical, morphological and genetics.

Morphological diagnosis resides in the alteration of dystrophin protein at immunofluorescence analysis in muscle biopsy sample. In Figure 2 there is an example of possible scenarios: the first image represents a healthy control. The dystrophin is correctly expressed and localized at the muscle membrane. The second image belongs to a BMD muscle specimen, where dystrophin-specific staining is not-homogenous, and signal intensity is greatly reduced. Finally, the muscle from a DMD patient clearly shows the complete absence of dystrophin protein; some revertant fibers (arrows) are stained. They are rare dystrophin positive fibers, present alone or in small clusters in roughly 50% of

DMD patients (Arechevala-Gomeza et al. 2010). In these dystrophin positive fibers, spontaneous exon skipping events lead to the omission of exons flanking the mutation with the aim to restore the correct reading frame. The resulting protein maintains N- and C-terminal domains and this allows its correct anchoring and localization (Thanh et al 1995).

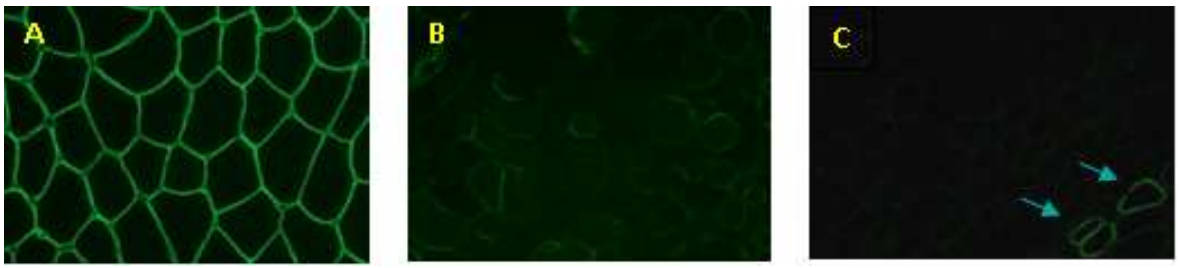

Figure 2 - Immunofluorescence of control muscle (A), BMD muscle (B), DMD muscle (C) (courtesy of Patrizia Sabatelli, Istituto Ortopedico Rizzoli, Bologna).

From the description given so far, it is clear that based on the entity of protein reduction or even absence, there will be different phenotypes: Duchenne muscle dystrophy (DMD) [OMIM 310200], Becker muscle dystrophy (BMD) [OMIM 300376], X-linked dilated cardiomyopathy (XLDC) [OMIM 302045], CPK elevation or isolated quadriceps myopathy, symptomatic carriers with various disease severities.
In this work, only DMD and BMD will be described. They are allelic disorders due to different mutations occurring in the same gene.

Duchenne muscle dystrophy: with an incidence of 1/5000 male births is the most severe phenotype and is characterized by complete dystrophin absence. The defect is present from birth but the clinical onset is postponed, around 12-24 months of age, with delayed motor milestones acquisition (boys start to walk around 18m on average) often associated with failure to thrive and intellectual disabilities (Bushby et al. 2009). Clinical diagnosis normally occurs at 2-3 yrs when movement impairment is more evident and the comparison with aged matched peers highlight difficulties in running, climbing stairs, standing up from a sitting or laying down position. In this case they use the typical Gower's manouvre: they stand up "climbing" by sustaining their weight with arms. DMD is a progressive disease and proceeds on until complete muscle wasting. A severe dilated cardiomyopathy often associated with arrhythmia invariably occurs. It is possible to delineate a typical evolution course of the disease: an initial phase in which motor milestones are reached, even if at a delayed time and with a top performance that is inferior

with respect to peers; a second variable lasting period of plateau followed by a relentless decline. In the late disease stage cardiac and respiratory impairment worsen, representing the two main causes of death (Merlini et al. 2015). Although relatively constant, disease evolution may vary among patients, depending on individual genetic background (genetic modifiers) and therapies.

Becker muscular dystrophy: allelic to Duchenne muscular dystrophy, although it has an incidence that is 1/5 with respect to DMD, this disease is highly prevalent due to its milder phenotype.

It is caused by milder mutations, both major "in-frame" rearrangements and small mutations in dystrophin gene, that don't abolish protein production (Fig. 2B) but induce quali/quantitative changes (Yagi et al. 2003) affecting muscle membrane stability.

Phenotypically the disease shares many characteristics with Duchenne muscular dystrophy but with a slower and milder progression: diagnosis generally occurs later, between 5 and 25 yrs and loss of ambulation not earlier than late teens. Life expectancy is slightly reduced and, depending on severity, they normally are able to reproduce, transmitting the mutated allele to the 100% of females. Cramps and muscle pain, mainly exercise related, are major symptoms, quadriceps muscles appear atrophic whereas calves showed paradoxic hypertrophy due to infiltration of fibrous adipose tissue. Dilated cardiomyopathy is also quite frequent in BMD and could be clinically more relevant with respect to body muscle involvement. Some BMD patients present cardiomyopathy as first symptom of the disease (Ferlini et al. 1999). Some BMDs recognize a differential diagnosis with other limb girdle muscular dystrophies (Manzur et al. 2009).

1.2. Current therapeutic approaches for Duchenne muscular dystrophy

The only recognized and approved drug for DMD are corticosteroids (prednisolone and deflazacort). These were approved both by FDA and EMA and recently added to the DMD gold standard therapies (AIFA has also recently aligned itself; http://www.agenziafarmaco.gov.it/content/fda-approvazione-accelerata-del-primo-farmaco-la-distrofia-muscolare-di-duchenne).

Corticosteroids have dramatically improved the disease course, primarily prolonging the ambulatory phase (loss of ambulation from 12 yrs to 18-20 yrs) and ameliorating cardiac and respiratory functions, although they are burden of some side effects that hamper their

application in the long run (Merlini et al. 2012; Angelini 2007; Hoffman et al. 2012; Lim et al. 2017).

Physiotherapy also represents a mandatory treatment for DMD. An appropriate and scheduled physiotherapy can be really efficacious in delaying the onset of tendinous retractions and scoliosis, helping in preserving respiratory function (Bushby et al. 2010).

Other symptomatic treatments such as ACE-inhibitor and beta-blockers are extensively applied but are efficacious only for the treatment of some symptoms and to prolong the physical and cardiac performance. Nevertheless the disease is still fatal and characterized by early mortality, to date life expectancy can reach 20-30yrs (Merlini, Sabatelli 2015).

Considering the severity and the prevalence of the disease many are the efforts in finding other therapeutic approaches: different strategies have been developed and some of them are currently in clinical trials. They can be divided in new drugs, aimed to treat the native dystrophin defect, or treatments that target secondary effect of the lacking protein. The first group comprises cellular therapy, gene therapy, RNA modulation therapy (including exon skipping and RNA interference and stop codon reversion).

Regarding cellular therapy it consists in the implantation of myogenic cells (ex vivo modified autologous or healthy heterologous) with the aim of promoting muscle regeneration and substitution with the new population finally obtaining new healthy muscle. Although applicable to all patients irrespectively of their mutation type, it has some disadvantages such as low implanted cells' survival, limited functional effects, graft versus host response, muscle specific targeting (Meng et al. 2016; Meregalli et al. 2013; Farini et al. 2009).

Gene therapy consists in the replacement of the defective gene with a functional one: huge dystrophin gene dimensions have made this approach really challenging. Mini or micro dystrophin have been set up (removing only dispensable domains) and introduced in different viral vectors but the efficacy of this treatment still remains a challenge depending on the low transfection efficiency, the unstable protein expression levels and the need of constant immunosuppressive therapy (Wang et al. 2017; Robinson-Hamm et al. 2016). Nevertheless, recent advances in research, trying to improve gene transfer capacity, has led this approach to land in a pilot trial in USA: a Phase 1/2a systemic gene therapy trial will investigate microdystrophin delivery in DMD; safety will be the primary outcome (https://musculardystrophynews.com/2017/01/09/hospital-researchers-awarded-2-2-million-duchenne-muscular-dystrophy-gene-therapy-investigation/).

Antisense oligonucleotides based therapy is currently in clinical trials but, it is mutation specific (as Ataluren, see below) and consequently cannot cure the totality of DMD population: this approach is expected to correct close to 78% of DMD deletion mutations (Niks, Aartsma Rus 2017). Different antisense oligonucleotides (AOs) compounds, with different chemistries, have been developed so far and, in particular 2′O-methyl phosphorothioate (2′O-MePS) and phosphorodiamidate morpholino oligomer (PMO) classes of AOs have been tested in clinical trials up to phase III. Although 2'O-MePS showed to be slightly more efficacious in inducing dystrophin restoration, they are burden of many side effects, mainly due to their charged backbone chemistry (Syed 2016; Lim et al. 2017). On the contrary, PMOs are safer mainly due to their lack of positive charge that makes them incapable to interact with proteins and more stable (Lim et al. 2017). Eteplirsen, a 30-nucleotide phosphorodiamidate morpholino oligomer (PMO), developed by Sarepta Therapeutics (Cambridge, MA, USA) has recently proven, based on surrogate end-point results, to be effective in increasing dystrophin level in DMD patients. Efficacy and tolerability data comes mainly from four clinical trials developed so far (NCT00844597, NCT01396239, NCT01540409, and NCT02255552) and recently (September 2016), the US Food and Drug Administration (FDA) approved this compound as the first and only available FDA-approved drug for DMD (Lim et al. 2017; US Food and Drug Administration. FDA grants accelerated approval to first drug for Duchenne muscular dystrophy [press release]. Silver Spring (MD): FDA; 2016 [September 19]. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm521263. November 18, 2016.).

The second drug which has received orphan drug designation and approval is Ataluren (Translarna, PTC Therapeutics) an aminoglycoside derivative, which is orally administered to treat patients affected by non-sense mutation (10-15% of the total DMD population)

The European Medicine Agency (EMA) has approved it as an orphan drug (http://www.ema.europa.eu/ema) and from 2016 AIFA (Italian Pharmacy Agency) deliberated its use with SSN (National Health Institute) with complete reimbursement. It leads to a translational read-through of nonsense mutation only, therefore the generated stop codon is replaced by a codon for amino acids allowing the protein formation to be completed. This is a great achievement for the Duchenne population and a very recent publication underlines the positive effect to ameliorate and delay the clinical progression of the disease and maintain the ambulation (McDonald et al. 2017).

Considering problems and limitations of new therapeutic approaches that resides mainly in efficacy and efficiency, recently the attention of researchers and companies has turned to traditional and already approved drugs that can be explored in terms of "drug repositioning", speeding up the approval process: generally these are well known pharmacological compounds with a toxic profile already delineated. These drugs are not only studied in terms of real treatments for the disease, but more realistically as compounds able to ameliorate disease course or even work synergistically with innovative drugs (De Luca 2012; Guiraud, Davies 2017).

Summarizing, remarkable improvements have been made on DMD therapies and new drugs are now available. Efficacy and tolerability profile are of course very relevant as well as identification of responders will facilitate drugs circulation, approval and recruitment in novel clinical trials. In this context there is the need to face the problem of correct trials design and also patients' stratification in order to obtain more reliable outcome data interpretation. Furthermore drug effects and possible toxic side effects need to be monitored and for this aim a profound knowledge of the disease is required (Hamuro et al. 2017). Finally, with the emerging data on pharmacogenomics, the possibility of a different response to different drugs in different subjects should be envisaged in order to avoid unexpected side effects and a priori failure of the treatment (Merlini, Sabatelli 2015; Aartsma Rus et al. 2017; Straub et al. 2016).

1.3. Biomarkers for rare disease

To overcome these problems, biomarkers could be an answer.

As defined by the National Institute of Health-NIH in 2001 a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic process, or pharmacologic responses to a therapeutic intervention or other health care intervention". According to European Medicine Agency (EMA) an ideal biomarker should be:

- a fingerprint, having analytical validity, and the estimated parameter must accurately and clearly discern between altered/normal status or treatment response/non-response of a patient. Specific tests used to identify biomarkers should be accurate, reliable and reproducible;
- a mirror, reflecting the disease or treatment in question, thereby providing validity. In other words, the ideal biomarker should document the activity or progression of the

disease and assess any effects of the treatment administered. In addition, it should not be influenced by other environmental factors such as age, sex, stress, diet exercise or other genetic determinants;

- a prophet, predicting, as an alternative to standard clinical assessments, disease progression (prognostic evaluation) or drug response (treatment evaluation), thereby representing a useful indicator of clinical outcome.

In addition it should be feasible (practical to measure and stable), non-invasive and time and cost effective (Scotton et al. 2014).

Biomarkers' types are genomic, proteomic and imaging ones. Genomic biomarkers include DNA and RNA marks, such as SNPs, CNVs, alleles (Hathout et al. 2016), specific transcripts isoforms or ncRNAs, miRNAs, methylated DNA. Their identification has greatly increased in recent years thanks to the adventure of highthroughput technologies that allow the analysis of a number of samples, genome-wide and at a high resolution.

Downstream to a genomic variation, generally an alteration in specific proteomic pathways can be identified: inside this deregulated networks proteomic biomarkers can be discovered. They have the added value to be easily accessible because they can be measured in body fluids (plasma, serum or urine) so they can be used for multiple sampling at different time points (disease or therapeutic monitoring). Their detection and quantification is relatively easy: immunoassays are now available, in particular based on multiplexes, and allow a sensitive identification of many proteins starting at low amounts of body fluids, thanks to the property of an antibody to react specifically with a protein (Figure 3). Enzyme linked immunosorbent assay (ELISA) is one of the most used technique: antibodies specific for the protein of interest are absorbed to a rigid support, then the body fluid is added; after antibody-antigen interaction the fluid is washed away and a second antibody, able to recognize the new antibody-antigen complex and linked to an enzyme, is added. The substrate for the specific enzyme then induces a chromogenic reaction; the final intensity is then measured by a spectrophotometer and the relative intensity correlates with the quantity of the protein explored (Butler 2008).

The recent growing interest in biomarkers discovery and validation is mainly due to their broad applicability and the need to optimize clinical trials' design and drug effect monitoring. Indeed, finding reliable biomarkers would facilitate not only disease correct diagnosis, but also disease monitoring and prognosis, patients stratification and prevision of individual response to drugs. Furthermore, sensitive and feasible biomarkers could be

used as primary outcome measures enabling an easier identification of drug effectiveness or, on the contrary, the onset of side effects, shortening the time of trials conduction and without exposing patients to unsolicited expectations.

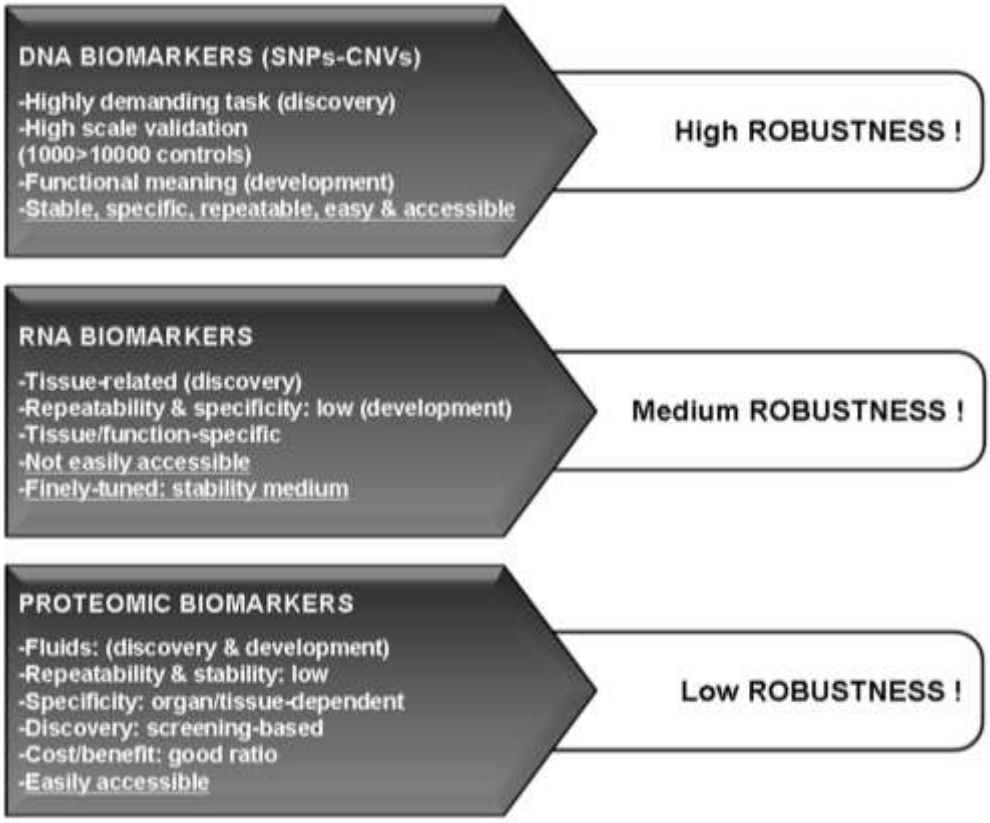

Figure 3 - Biomarkers main characteristics. Different pros and cons are considered and listed (from Scotton et al. 2014).

Many different approaches have been developed and applied to date for biomarkers' discovery and validation. With the advent of highthroughput technologies, omics studies have drawn increasing interest, showing a high detection rate despite difficulties in interpretation an validation of such a huge amount of generated data. A possibility to overcome the problem is the approach by candidate that implies a preliminary selection, made in silico, searching into available data on diseases' pathways. A really promising prospect is the combination of this two, the so called "omics by candidate" research. A brilliant example has been described by Kotelnikova et al.: developing various statistic strategies based on in-silico tools (Limma, Pathway studio), they crossed publicly available expression array data on DMD with literature information about main disrupted networks in the disease, with the aim of to identify possible biomarkers and drug targets to

be further investigated (Kotelnikova et la. 2012). The rationale resides in the "screening" of differential expressed genes, between control and samples, their assembling based on Gene Ontology groups and the subsequent prioritization of some candidates based on their role as key regulators in different pathways (Scotton et al. 2013). This is one of the most promising approaches and probably the one that could combine relatively sustainable costs along with a high detection rate.

An extrapolation of this approach has been used in the present work and will be described below.

1.4. Finding a model for pre-clinical studies: the mdx mouse

Recent experience in clinical trials for rare diseases, for which Duchenne muscular dystrophy could be considered paradigmatic, has highlighted many problems as above mentioned, in particular in terms of high demanding task for patients and their families, their exposure to side effects and costs in setting up clinical trials for drugs that finally revealed to be ineffective. For this reason there is a sort of "back to the origin" trend, both for academic and private research, with a renovated interest for *in vitro* basic research and animal model experimentation. This latter poses many problems, not only because of high demanding in terms of people and costs, but also because it is challenging to identify suitable animal models that could be used for clinical translation of results.

The dystrophin gene is highly conserved among vertebrates. Many animal models of dystrophinopaties exist and they are broadly used to study muscle pathology and drug treatment effects (mouse, golden retriever, C. elegans, zebrafish).

One of most known is the mdx mouse, first described in 1981 (Bulfield et al., 1981); it carries a point mutation in exon 23 (C>T at nucleotide 28) leading to the onset of a stop codon instead of a glutamine. Although dystrophin absence, the overall phenotype of this mouse is less severe with respect to Duchenne muscular dystrophy (Lynch et al. 2001): the lifespan is reduced 20% with respect to *wt*, and there is an abrupt onset of muscle degeneration and necrosis starting at 3-4 weeks that reaches its maximum at 8-12 weeks of age which then becomes chronic with a further reduction at about 1 year (Grounds et al. 2008). Adult mdx doesn't show great function impairment until 1 year of age and fibrosis becomes more evident at around 15 months (Muntoni et al. 1993; Lefaucheur et al. 1995). Different stages of dystrophin related pathology are present in different muscle types with different severity: the most affected are hind limb muscles (tibialis anterior, gastrocnemius,

quadriceps, soleus, extensor digitorum brevis the most studied) and diaphragm, this latter is probably due to continuous respiratory contractions. Other muscle groups, such as masseter or laryngeal muscles are relatively spared from myo-nechrosis, which is the reason why it is not completely understood (Ground et al 2009).

The milder phenotype present in mdx may reside in part in the up-regulation of utrophin, a dystrophin homologue, that is expressed in humans during all the fetal period and during muscle regeneration. Utrophin is expressed in adult muscle where is present only in myotendineous and neuromuscular junctions (Zhang et al 2009). On the other hand, the mdx mouse presents in muscle histology high proportion of dystrophin positive revertant fibers, which also represents a problem for efficacy evaluation of preclinical drug studies (Pigozzo et al. 2013).

Although broadly used in preclinical studies for dystrophinopaties it doesn't seem to be the gold standard clinical model (Zhang et al. 2009).

Exercise may modify skeletal muscle pathology in mdx mice, with a different impact in different muscle types, contributing to worsening the overall muscle phenotype, prolonging the degenerative phase and enhancing patients' like alterations (Ground et al. 2009; Camerino et al. 2014; TREAT-NMD, www.treat-nmd.eu).

Exercise regulates skeletal muscle function, morphology and metabolism. In response to different types and schedules of the exercise load there is the activation of different molecular pathways in order to correctly answer this request. The balance is important because, even if exercise is one of the main functions of muscle and is known for its beneficial effects, strong exercise could be harmful. In DMD the role of exercise in muscle damage is poorly understood.

Experiments in mdx mice with different exercise protocols suggest a reduced threshold to muscle damage induced by contraction. Voluntary exercise seems to be positive, on the contrary forced or strong exercise leads to an exacerbation of dystrophic phenotype in mdx (Hayes et al. 1996; De Luca et al. 2003). In addition there is growing evidence that the same exercise protocol doesn't have the same impact on all muscle types. For example 48 hours of voluntary wheel running largely worsens quadriceps phenotype in mdx, with less effects in TA and diaphragm muscle (Archer et al. 2006). The molecular mechanisms under these events are unknown.

Camerino and co-workers (2014) used a protocol of forced horizontal treadmill running as exercise stimulus for mdx mice: at least 4 weeks of exercise worsened the phenotype to a

more DMD similar one. Exercised mdx show enhanced weakness and fatigability with respect to *wt*, more pronounced muscle necrosis and fibrosis, elevated plasma levels of calcium and muscle enzymes as well as markers of oxidative stress.

They found an up regulation of Pcg1a (AMPK pathway) that ameliorates mdx phenotype inducing a fast to slow switch on fiber types, reduction of oxidative stress and utrophin upregulation. The same effect was seen via the activation of upstream or downstream signals such as SIRT1. These data support the hypothesis of a role of the fast to slow fibers switch in contributing to stabilize the mdx muscle degeneration after the first acute period explaining the milder phenotype of mdx.

In general a fast to slow switch in *wt* in response to chronic exercise is known. The exercise protocol described in the study revealed not to be sufficient to induce this type of change in *wt*, but in mdx they found an upregulation of Sirt1 pathway that is a key regulator of the switch. Exercise on the contrary showed to revert this trend particularly in longer training, supporting the hypothesis that in DMD there is a maladaptation to exercise. Indeed a failure in mitochondrial oxidative metabolism is known in DMD patients in response to exercise and this may explain this impairment. Again, a down regulation of anti-inflammatory response and autophagy pathway were observed. Finally they confirmed that in dystrophic muscle there is a maladaptation to mechanic-transduction signals that drives to cumulative damage and is more severe in fast fibers. Which explains why some muscles are more involved than others (muscle fibers composition and function).

Considering all these evidences, scheduled exercise is good for phenotype worsening in mdx creating a model similar to DMD and is more suitable for pre-clinical drug studies. This will allow to obtain more robust pre-clinical results in order to pass to clinical applications more promising therapies (Camerino et al., 2014).

1.5. Circadian rhythm pathway: definition and its role in skeletal muscle

Time is a fundamental element in life: it regulates many activities in all organisms, from monocellular bacteria to plants and animals (Gaudi et al. 2000, Schroder and Esser 2013). It can be divided in linear and circular, and the latter can be characterized by a peculiar rhythmicity (Gaudi et al. 2000).

The most relevant is the rhythm that follows the characteristic 24 hours daily period, named circadian rhythm: it has been extensively studied in chronobiology and leads to a

specific pattern of molecules' oscillation with a periodic quali/quantitative change in both transcriptome and proteome all around the body. Its correct functioning is fundamental for the synchronization of different cellular activities in response to external cues (light/dark, food ect.) allowing for a more efficient and energetically safe adaptation of the organism (Ko and Takahashi, 2006). In humans, an example of body adaptation to external stimuli are circadian oscillation in body temperature, blood pressure, cardiac frequency, cortisone blood levels etc. Studying how different external cues may influence rhythmicity is fundamental so as to correctly understand, how circadian pathway works on one hand, and on the other how they may synchronize each other (Ko et al. 2006).

Circadian rhythm is determined by a network of self-sustaining transcriptional-translational loops (Figure 4). This network is phylogenetically highly conserved and consists of positive effectors inhibited by negative ones.

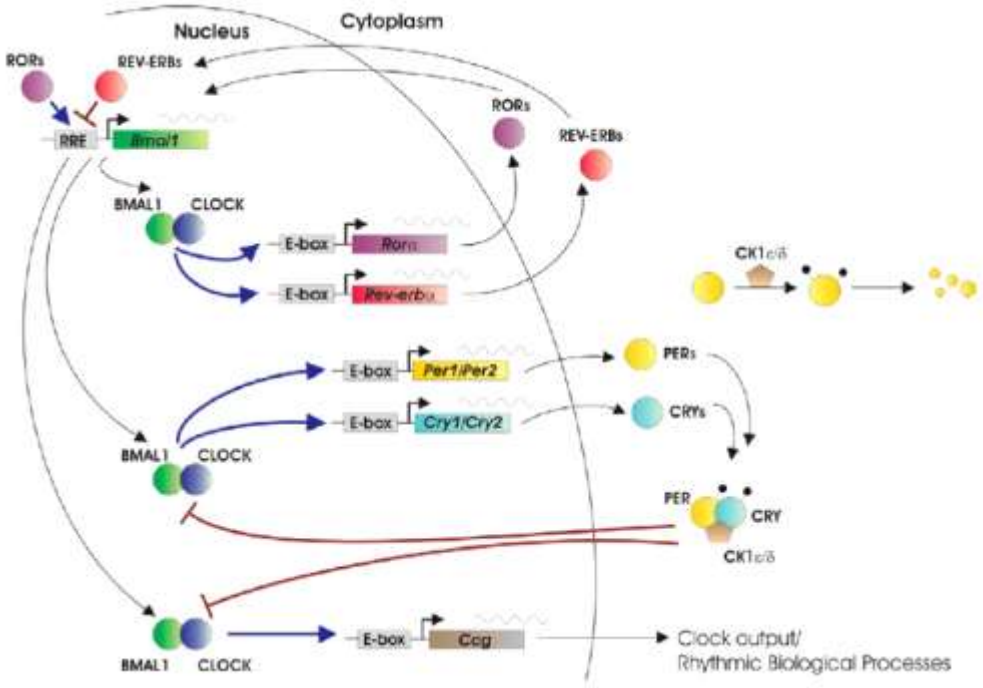

Figure 4 - Circadian rhythm pathway consists of a complex system of self-sustaining transcriptional-translational feed-back loops (Ko et al. 2006).

This pathway in mammals is composed by the heterodimer formed by PAS-bHLP proteins CLOCK and ARNTL1, that binds to different E-box DNA elements, enhancing the transcription of many effectors genes (output) the most relevant of which are Per and Cry proteins that constitute the negative arm of the loop. CLOCK protein doesn't change their level during the 24 hours cycle, on the contrary ARNTL1 has a circadian pattern of expression. Together with them, also ROR and Rev-erb family members exert an important

role in maintaining circadian rhythmicity, the first activating and the second inhibiting ARNTL1 specific transcription (Schroder and Esser 2013). These circadian clock core components mainly activate transcriptional factors of different genes involved in as many as cellular functions such as cell cycle, cellular metabolism, regeneration, protein degradation. Part of the master timekeeping network are also different molecules with different enzymatic activities that regulates post transcriptional and translational modification of core clock genes and act mainly in determining the phase of circadian oscillation around the 24 hours. Casein kinase 1δ, for example phosphorilates Per1 and mutations in it or in the phosphorylation site of Per1, have been associated to familial advanced sleep phase syndrome (OMIM 615224); Sirt1 and Cry, on the contrary, have a role in histone acetylation, the first deacetylates histone H3, the latter, in association with p300, promotes acetylation.

In multicellular organisms such as mammals, rhythm pathways are characterized also by the presence of a pacemaker that promotes the synchronization of all tissues so they can work in a coordinated fashion. In mammals this master regulatory element resides in the suprachiasmatic nucleus (SCN) in the anterior part of the hypothalamus. It is directly linked to the retina and other receptors all over the body, and, via hormones release, it can stimulate different and coordinated adaptive responses to external stimuli. Different SCN ablation experiments conducted in animals (hamsters and rats) have demonstrated that the absence of this coordinator leads to a desynchronization and a general phase shift in molecular circadian expression patterns and consequently a change in normal periodicity of daily activities such as sleep, liquid intake and exercise (Stephan et al., 1972).

The presence of self-sustaining rhythmicity in peripheral tissues despite SCN regulation was discovered about fifteen years ago: different core clock genes may act in different ways in different tissues, and their interactions could be tissue-specific and independently influenced by external stimuli. This interesting observation poses the question if a sort of tissue-specific circadian subnetworks may exist, which could pave the way for studying circadian regulation and rhythmicity in singular tissues such as liver, kidney, lung and skeletal muscle (Wolff and Esser 2012).

Skeletal muscle has been intensively studied in recent years because of its great importance both in health and disease state. Muscle contributes for 45% of all body mass, is the first reservoir of amino acids and glycogen for glucose production and mobilization during fasting periods and exercise, in tight collaboration with liver, and could act as an

endocrine tissue via myokines secretion (Harfmann et al. 2015). It is also involved in many highly prevalent human diseases such as cancer, cardiovascular disease or type 2 diabetes, where muscle insulin resistance is a key step in pathogenesis (Harfmann et al., 2015, Louchart et al. 2015).

The first skeletal muscle expression profiling study by McCarthy and colleagues in 2007, identified 215 transcripts with circadian expression rhythmicity, belonging to GO groups of metabolism, transcription factors, protein degradation, apoptosis and regeneration. They also compared transcript change between *wt* mouse and Clock KO, finding that around 35% of the genes were differentially expressed, supporting the hypothesis of the great relevance of circadian pathway control in muscle physiology. Indeed, an indirect proof could be inferred observing the muscle phenotype in circadian animal models: $Clock^{\Delta 19}$ and $Bmal/Arntl1^{-/-}$ mice's skeletal muscle is broadly affected showing a 30% reduction in maximal force and tissue structure. The sarcomere organization and protein content is largely subverted and in $Clock^{\Delta 19}$ a decrease in Pgc1a protein and an overall reduction in mitochondria content have been observed (Andrews et al., 2010; Harfmann et al. 2015). Interestingly, Schroder and colleagues (2015) showed a strength reduction, fast to slow switch and increased muscle fibrosis associated with a change in TGFβ factors superfamily in selective $Arntl1^{-/-}$, this latter showing also overall features of muscular dystrophies, myopathies and ageing. The change in phenotype occurred in absence of other interfering cues such as light, exercise or feeding. So they concluded that the specific disruption of Arntl1 cause alteration of gene expression relative to altered activation of transcription factors (Schroder et al. 2015).

Technical advance in transcriptomics analysis has led the number of mRNA with circadian expression to rich 2300, among them many muscle specific such as MyoD, MyoG, Ucp3, FbxO32. MyoD and MyoG are characterized by a circadian expression. They are bHLH transcriptional factors, expressed only in skeletal muscle and drive muscle differentiation and regeneration. MyoD is expressed in the very early differentiating phase and contains a specific non-canonical E-box element for a direct interaction with Clock-Arntl1 heterodimer (Andrews et al., 2010; Zhang et al. 2012); MyoG is expressed only in late phases and is a downstream effector of MyoD. The identification of MyoD as a Clock Controlled Gene (CCG) suggests a mechanism by which circadian clock pathway may regulate muscle specific daily transcription driving adaptation to internal and external stimuli (Andrews et al., 2010; Zhang et al. 2012).

Considering the emerging role of circadian rhythm in muscle homeostasis, there are many evidences that external stimuli, such as light, feeding and exercise, may influence muscle circadian transcription. In particular, exercise has been proven to act directly on the molecular clock in humans by Zambon and collaborators via modulation of Per2, Cry1 and Arntl1 expression. They showed for the first time that not only resistance exercises may influence circadian rhythmicity, but also that the specific schedule of exercise adopted is of great importance. Exercise drives the activation and expression of many genes that are particularly involved in specific skeletal muscle metabolic pathways: AMPK and PGC1α seem to be two of the key regulators of these interactions. In these terms, physiological response to exercise differs in relation to the specific time of the day in which it is performed: the best sport performance in humans is reached when training is done daily at the same time because the molecular clock can synchronize and modulate activation of muscle specific exercise pathways favoring correct metabolic ways and limiting muscle damage (Murphy et al., 2014). More recently, Wolff and Esser (2012) highlighted the effects of scheduled exercise in modulating the phase of circadian clock. Studying three different muscle types, they demonstrated the presence of a peak of expression of Per2 protein in skeletal muscle but not in SCN, suggesting a specific synchronizing effect of exercise on peripheral tissues. Interestingly at baseline they showed a different circadian expression profile in the three different muscle types: this is the first time in which the possibility of a specific oscillation related to muscle type has been observed opening the way for studies aimed to characterize the specific circadian transcriptome for muscle respect to fiber type composition embryological origin and specific mechanic function (Wolff and Esser 2012).

1.6. Circadian rhythm genes and muscle disease: the collagen VI myopathies example

The tight link between circadian clock and muscle has recently been further delineated in our work about the new discovery of circadian genes deregulation in collagen VI related myopathies (Betlhem myopathy-BM and Ullrich congenital muscular dystrophy-UCMD).
In this work comprehensive expression profiling of both col6$^{-/-}$ mouse model and BM/UCMD patients allowed us to identify, among other better known molecular alterations (inflammatory network, apoptosis, transcriptional regulation, transport), a profound deregulation of clock component genes, CLOCK for first. In this work Scotton

and colleagues (Scotton et al. 2016), applied an expression-array based approach on collagen VI deficient mouse model, and unraveled that Arntl1, Clock, Per1 and 3 are deeply deregulated in three different muscles types (gastrocnemius, tibialis anterior and diaphragm). The data have been confirmed using a customized low density Taqman based assay containing 46 key regulator genes selected among the most deregulated genes identified by our array study followed by SNEA analysis. The rhythmic genes Arntl1, Atf5, Clock, Dbp, Egr1, Fkbp, Per1, Per2, Per 3 and transcription factors relevant for muscle remodeling and regeneration (MyoD1, MyoG and MYF6) were found deregulated in all three muscles; protein quantification study by western blot in collagen VI KO mouse model *Col6a1*$^{-/-}$ identified an increase of clock protein compared to *wt* sample. RNAseq performed in collagen VI patients (1Ullrich congenital muscular dystrophy and 2 Betlhem myopathy) showed the same circadian rhythm deregulation and Real-time analysis of CLOCK and MAT2A showed a profound down regulation in an enlarged cohort of patients (11 UCMD and 12 BM). Western blot analysis in 4 UCMD patients and 3 BM confirmed the presence of an increased quantity of clock protein in UCMD samples (Scotton et al. 2016).

These completely new findings showed for the first time an involvement of circadian rhythm pathways in a severe hereditary myopathy. According to the possible dual interpretation given by Scotton et al., who hypothesized both a downstream effect of CCG in collagen VI myopathies and an independent possible disease modifier mechanism, the identification of deregulation of rhythmic process in primary muscle disease suggests that further analyses are needed to explore the meaning of circadian pathway in genetic disorders. The possibility to use circadian signatures as biomarker and therapeutic target deserves further studies.

2. AIM OF THE STUDY

Based on the recent findings showing the profound deregulation of this pathway in the severe collagen VI related myopathies, and the growing body of data about a circadian regulation of gene expression in skeletal muscle, the aim of the present study was to explore the possible involvement of circadian rhythm in the physiopathogenesis of Duchenne muscular dystrophy.

For this purpose, transcriptomics studies in skeletal muscle from both animal model (*mdx*) and patients were carried on in order to underline the altered pathways involved in the disease. This may allow discovering of new promising biomarkers to be further explored both for diagnostic and therapeutic perspectives.

Furthermore, considering the relevance of both topics, circadian circuit and Duchenne disease, discovering a link could open new ways in terms of chronotherapy, namely the correct therapy at the right moment, greatly impacting therapeutic options for this severe disease, through an optimized personalized treatment.

3. MATERIALS AND METHODS

3.1. Circadian genes expression analysis by custom Fluid-CIRC Taqman based assay in mdx mice

3.1.1. Animal selection and RNA extraction

In vivo experiments and the animal housing was performed according to the Italian law for Guidelines for Care and Use of Laboratory Animals (D.L. 116/92), and the European Directive (2010/63/UE) at the Department of Pharmacobiology, Faculty of Pharmacy, University of Bari, Bari, Italy, and performed by the group of Prof. Annamaria De Luca (University of Bari). Experimental procedures were conducted accordingly to standard operating procedures for pre-clinical test in mdx mice available at TREAT-NMD website (http://www.treat-nmd.eu/reasearch/preclinical/dmd-sops/).

In this study a total of 3 wild type (WT; C57/BL10ScSn) and 27 mdx male mice at the age of 4-5 weeks (IFFA Credo and Jackson Laboratories) and homogeneous for their body weight were selected. The mdx mice were divided into two groups the sedentary group, which consist of 3 mdx mice, and the exercised group, which consist of 24 mdx mice. The exercise protocol was performed as previously described (Camerino et al. 2014) by running for 30-min on a horizontal treadmill (Columbus Instruments) at 12 m/min, twice a week (keeping a constant interval of 2–3 days between each trial), for 4–8 wk. A total of 20 exercised mdx mice were treated one day before the beginning of the exercise protocol with 7 different drugs (see Table 1) as previously described (Burdi et al. 2009. Cozzoli et al., 2011, Camerino et al. 2015, Capogrosso et al. 2016) (Table 1) and the treatment was continued until the day of sacrifice. Both WT and "sedentary" mdx mice were left to move freely in the cage without any exercise. Both exercised and sedentary mice were monitored at the same time points.

Two types of muscles, Gastrocnemious (GC) and Tibialis anterior (TA) were collected at 8–12 weeks and 16-20 weeks respectively, from WT and mdx mice related to the different groups, in the same time window, after intraperitoneal anesthesia using 1.2 g/kg urethane, and were washed in PBS and rapidly frozen in liquid nitrogen-cooled isopentane and stored at -80°C until use.

All RNA and protein studies on these mice were performed by medical Genetics Unit at the University of Ferrara group.

Total RNA was isolated using RNeasy-kit (Qiagen, Chatsworth, CA) according to manufacturer's instructions, and treated twice with DNase (RNease free DNAase set

Qiagen kit) to exclude possible genomic contamination. DNA contamination check was perfomed by using a Real-time PCR system designed on murine actin B. Nucleic acid concentration was quantified using Nanodrop (Thermo Scientific) spectrophotometer.

Mice cohorts		Gastrocnemius (Age 8-12w)	Tibialis anterior (Age 16-20)
wt		3	3
mdx untreated	mdx sedentary	3	3
	mdx exercised + vehicle (95% water)	3	3
mdx treated	mdx exer + alpha methyl predinisolone (i.p.)	3	
	mdx exer + Resveratrol 100 mg/kg (i.p.)	3	
	mdx exer + Pentoxifylline 50 mg/kg (i.p.)	3	
	mdx exer + Nandrolone 5 mg/kg (s.c.)	3	
	mdx exer + Taurine 1g/kg (os)	3	
	mdx exer + Apocynin 38 mg/kg (os)	3	
	mdx exer + Enalapril 5 mg/kg (i.p.)	2	

Table 1 - Mdx mice cohorts. In the "treated" column dosages for each drug are detailed.

3.1.2. Custom Fluid-CIRC design and circadian genes expression analysis

To characterize the involvement of circadian rhythm in the severe muscle wasting Duchenne muscular dystrophy, we selected a total of 30 murine genes (Table 2), following an accurate selection and prioritization based on the state of art reported in literature. The identified genes are involved in circadian rhythm, muscle regeneration, metabolism, apoptosis, immune reaction and cellular proliferation. We also included some candidate biomarkers identified by us and reported in Kotelnikova et al. (2012). They were selected to set up *ad hoc*, and, a custom low density micro-fluidic card TaqMan based assay (TLDA), different from the one described in Scotton et al. 2016, named Fluid-CIRC: it is a 384 wells plate pre-loaded with TaqMan gene expression assays (Applied Biosystems, Foster City, CA) and allows the run of 1 to 8 samples per card for 12 to 384 TaqMan gene expression targets. The design has been chosen among different available and the expression assays to be included have been selected from Applied Biosystems's

inventoried ones. In the chosen design all genes are run in triplicate, and 4 samples were run in each card; 18s was included by default in the design, instead Actin-B and Gapdh were included as additional references (Figure 5). Considering both the number of samples that can be run per card and the expression assays number, this tool is time consuming and requires a very small amount of both samples and reagents, providing a more sensitive and reproducible test.

With this tool we meant to obtain a transcriptional profile of selected genes in all the mdx enrolled in this study, both sedentary and exercised mice, and treated with different drug compounds by the analysis of the above mentioned selected muscles.

Figure 5 - Fluid-CIRC low density TaqMan based assay design.

	Gene code	Gene name	Function
1	**Arntl1**	Aryl Hydrocarbon Receptor Nuclear Transclocator-Like1	Transcription factor, core clock genes' component of positive loop
2	**Arntl2**	Aryl Hydrocarbon Receptor Nuclear Transclocator-Like2	Transcription factor, core clock genes' component of positive loop
3	**Atf5**	Activating Transcription Factor 5	Transcriptional repressor; it blocks the differentiation of neuronal progenitor cells
4	**18s rRNA**	ribosomal RNA 18s	Component of 40s minor subunit of ribosome
5	**Ccrn4l**	Carbon Catabolite Repressor 4-like (Nocturnine)	Deadenylase; it plays an important role in post-transcriptional regulation of metabolic genes under circadian control.
6	**Clock**	Circadian Locomoter Output Cycles Kaput	Core clock gene, involved in the positive arm of the transcriptional-translational feedback loop.
7	**Dbp**	D Site Binding Protein	Transcriptional activator, not crucial

			for circadian rhythm but modulates important clock output genes
8	**Egr1**	Early Growth Response 1	Transcriptional regulator
9	**Fkbp5**	Fk506 Binding Protein 5	Regulator of trafficking of steroid receptor containing vesicles
10	**Per1**	Period circadian clock 1	CCGs component of the negative transcriptional-translational regulatory negative loop
11	**Per2**	Period circadian clock 2	CCGs component of the negative transcriptional-translational regulatory negative loop
12	**Per3**	Period circadian clock 3	CCGs component of the negative transcriptional-translational regulatory negative loop
13	**Cry1**	Cryptochrome Circadian Clock1	Transcriptional repressor of circadian positive loop. It translocate Per proteins into the nucleus
14	**Cry2**	Cryptochrome Circadian Clock1	Transcriptional repressor of circadian positive loop. It translocate Per proteins into the nucleus
15	**Rorα**	RAR-Related Orphan Receptor A	Transcriptional factor that regulates lipid metabolism, circadian rhythm and skeletal muscle differentiation
16	**Nr1d1**	Nuclear Receptor Subfamily1, Group D, Member 1	Transcriptional repressor of Clock, Arntl1, Cry1
17	**Nr1d2**	Nuclear Receptor Subfamily1, Group D, Member 2	Transcriptional repressor of Clock, Arntl1, Cry1
18	**Csnk1ε**	Casein Kinase 1, Epsilon	Kinase that phosphorylates many proteins, among which circadian proteins Per1 and 2
19	**Csnk1δ**	Casein Kinase 1, Delta	Kinase that phosphorylates many proteins, among which circadian proteins Per1 and 2
20	**Bhlhe40**	Basic Helix-Loop-Helix Family, Member E40	Transcriptional Factor. Interacts with Arntl, and indirectly modulates Per1 transactivation via Clock/Antl1
21	**Bhlhe41**	Basic Helix-Loop-Helix Family, Member E41	Transcriptional repressor
22	**Tim**	Timeless circadian clock	Transcriptional repressor of circadian genes involved in the positive loop
23	**Sirt1**	Sirtuin1	Deacetylase involved in many different functions such as: DNA repair, metabolism, apoptosis, autophagy.
24	**MyoD1**	Myogenic Differentiation 1	Transcriptional activator of muscle specific genes mainly involved in muscle differentiation. It regulates myogenesis
25	**MyoG**	Myogenin (Myogenic factor 4)	Transcriptional activator of many muscle specific genes. It plays a role in end-stage muscle differentiation and adult muscle phenotype

26	**Dmd**	Dystrophin	Muscle specific structural protein
27	**Ppargc1α**	Peroxisome Proliferator Activated Receptor Gamma, Coactivator 1α	Transcriptional coactivator of steroid and nuclear receptors, role in fat acids and glucose metabolism
28	**Tgfb1**	Transforming Growth factor, Beta 1	It controls cellular proliferation and differentiation
29	**Gapdh**	Glyceraldehyde-3-Phosphate dehydrogenase	Role in glucolisis, transcription, RNA transport, DNA replication and apoptosis
30	**ActB**	Actin, Beta	Globular protein, it forms thin filaments of sarcomere

Table 2 - Selected genes for Fluidic-Circ design. Genes' specific functions are listed.

For each muscle sample, of all mice cohorts, a total of 300ng of RNA were retrotranscribed using High-capacity cDNA reverse Transcription Kit (Applied Biosystems) and then added with 100µl of Real-time Universal PCR Master mix. An amount of sterile water to reach a total volume of 200µl was added and the final solution loaded in 2 ports (100µl each) of the Fluid-CIRC and run on ABI 7900HT System Fast Real-time PCR System (Applied Biosystems) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles at 97°C for 15 sec, 1 min at 60°C (Applied Biosystems TaqMan Array Micro Fluidic Cards user Guide) (Figure 6).

After normalization of each experiment with Actin-B as housekeeping reference, data were analyzed following the $2^{-\Delta\Delta Ct}$ method, using *wt* samples as controls. Considering that the samples were run in different experiments, in order to optimize the comparison, we calculated the average of ΔCt for each muscle sample and used this to infer the final $2^{-\Delta\Delta Ct}$ for each gene.

$2^{-\Delta\Delta Ct}$ method allows the relative quantification of the expression of a target gene with respect to a control and is measured considering how much the $2^{-\Delta\Delta Ct}$ value moves away from 1 (case=control): <1 under-expression, >1 overexpression (Livak et al. 2001).

Mice were subdivided into 3 cohorts, wild type (*wt*), mdx, exercised (*mdx-exe*) or not (*mdx*), and mdx exercised+treatments (*mdx-exe+treatment*), for the two muscles (GC and TA) and were compared according to the following schedule: mdx vs *wt*, mdx-exe vs mdx, mdx-exe + treatment vs *wt*. For this latter, only the 7 prioritized genes have been analyzed (see below).

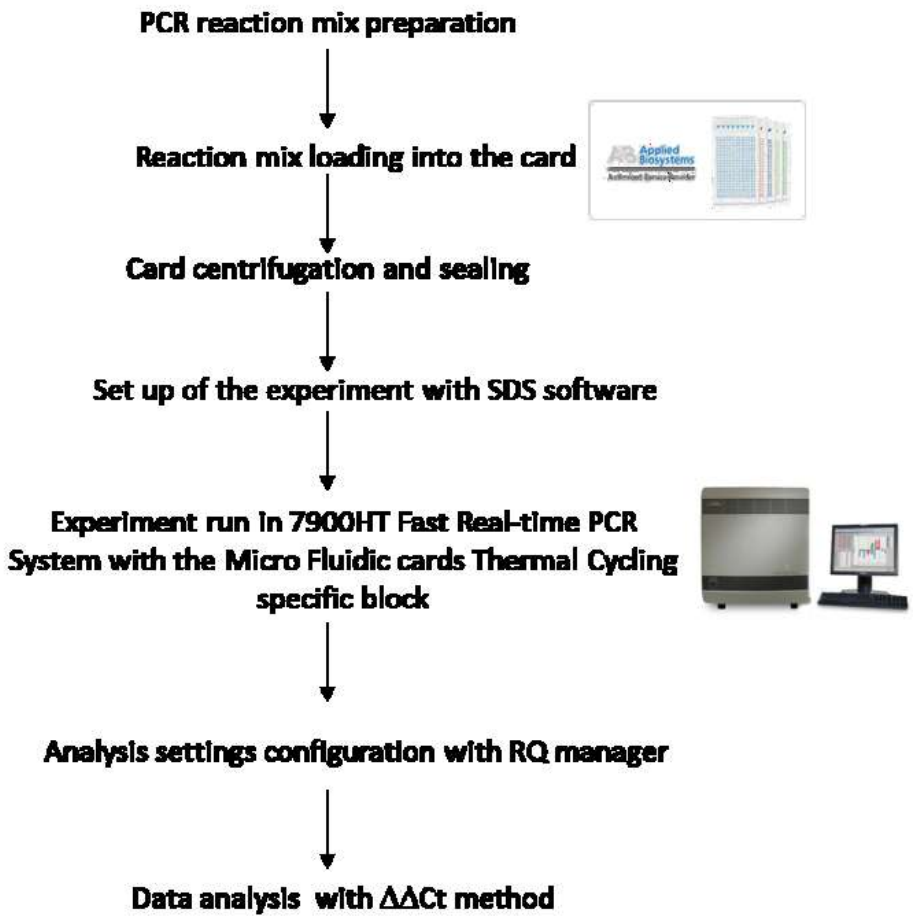

Figure 6 - Fluidic card flow chart (Applied Biosystems technical bulletin).

3.2. Most deregulated genes prioritization

Based on Fluid-CIRC expression data, Elena Schwartz and collaborators (Ariadne Genomics, Rockville MD, USA) performed statistical analysis, and prioritized the most deregulated genes among those analyzed in order to identify possible biomarkers to be explored in humans. In brief, they used an algorithm of statistical analysis extrapolated from Kotelnikova et al. 2012: using sub-network enrichment analysis (SNEA) potential transcription regulators/key proteins among the deregulated genes were identified and implemented in Pathway Studio. This approach allowed us to evidence nodal molecules and their downstream effectors concordant in expression change among networks mainly involved in circadian rhythm circuitry and DMD pathogenesis. Information about gene-gene regulation and protein-protein interaction were derived from available literature and stored in Pathway Studio. Seven genes (csnk1e, sirt1, myog, myod, cry1, cry2, arntl) were prioritized as most relevant for their deeply changed expression profile.

3.3. Expression analysis of prioritized genes in DMD patients' muscle

3.3.3. Patients selection, RNA extraction and Real-time PCR analysis

Considering the data obtained in the mdx mice model, to study a possible deregulated expression of the 7 selected genes (csnk1e, sirt1, myog, myod, cry1, cry2, arntl) in humans, we enrolled 9 DMD patients, with different mutation types. Mutations and clinic characteristics are detailed in Table 3. From each DMD subject we obtained muscle biopsy after informed consent and approval from the S. Anna University Hospital of Ferrara's Ethic Committee (no. 02/2009, 26th February 2009). This research has been conducted following the declaration of Helsinky rules concerning human subject research. Commercial control healthy muscles were used as reference (Tebu-bio, www.tebu-bio.com).

Muscle samples collecting procedures were carried out following local standard surgical procedures at a time window between 8-10 am (CET) and frozen shortly after collection in liquid nitrogen, until use. The concordance of collection time for each sample allowed us to correctly analyze and compare molecular clock component genes.

Total RNA was isolated from DMD patients' and controls muscle specimens using RNeasy-kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions and double treated with DNase (RNease free DNAase set Qiagen kit) to minimize possible genomic contamination. DNA contamination check was performed using a Real-time PCR system designed in intron 14 of the dystrophin gene. Nucleic acid concentration was quantified using the Nanodrop (Thermo Scientific) spectrophotometer.

Extracted RNA was reverse transcribed using High Capacity cDNA reverse transcription kit (Applied Biosystems); RNA from 3 healthy donors was pooled and retrotranscribed in order to obtain the same quantity of DMD samples. Transcript quantification was obtained using commercially available TaqMan expression assays (Applied Biosystems): CSNK1E, NM_001894.4, Hs00266431_m1, exon boundaries 6-7; SIRT1, NM_001142498.1, Hs01009006_m1, exon boundaries 7-8; MYOG, NM_002479.5, Hs01072232_m1, exon boundaries 2-3; MYOD1, NM_002478.4, Hs02330075_g1, exon boundaries 1-2; CRY1, NM_004075.4, Hs00172734_m1 exon boundaries 2-3; CRY2, NM_001127457.1, Hs00323654_m1, exon boundaries 5-6; ARNTL, NM_001030272.1, Hs00154147_m1, exon boundaries 8-9.

Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method with ACTIN-B as the housekeeping gene and pooled RNA from 3 healthy donors as control.

Patient	Mutation	Phenotype	Age at sampling
PT1	Deletion exons 46-55	DMD	13 yrs
PT2	Deletion exons 61-63	DMD	11 yrs
PT3	Duplication exons 5-7	DMD	11 yrs
PT4	c.2950-2A>G	DMD	7 yrs
PT5	c.9808-1G>A	DMD	4 yrs
PT6	c.3655-3656indelGG>TT, p.E1150X	DMD	4 yrs
PT7	c.2510C>T, p.R768X	DMD	8 yrs
PT8	c.8027+2T>A	DMD	4 yrs
PT9	c.10223+2T>C	DMD	4 yrs

Table 3 - Duchenne patients selected for expression analysis of the 7 most deregulated genes. Age at muscle sampling and mutations are detailed.

3.4. Casein kinase 1 epsilon (CSNK1E) protein quantification in plasma of DMD and BMD patients using Enzyme-Linked Immunosorbent Assay (ELISA)

3.4.4. DMD and BMD patients selection and plasma isolation

Plasma samples from other 16 patients, 2 BMD and 14 DMD, and 5 healthy donors were collected after informed consent and approval of the Ethics Committee of S.Anna University Hospital of Ferrara (no. 02/2009, 26 Feb. 2009, BIO-NMD European Union Seventh Framework Programme). Genotypic and phenotypic information are summarized in Table 4.

Plasma was isolated from peripheral blood after a single centrifugation, within 2 hours after sampling, at 1500g for 10 min at 4°C. The supernatant (plasma) was removed without disturbing the pellet and divided in aliquots of 400 µl each and stored at -80°C until use.

Code	Mutation	DMD/BMD	Age at loss of ambulation	Age at plasma sampling
A	del exons 3-7 (out of frame)	BMD	20yrs	33yrs7m

B	del exon 13	BMD	Ambulant at sampling	7yrs2m
C	del exon 43	DMD	Ambulant at sampling	8yrs
D	del exon 45	DMD	Ambulant at sampling	9yrs
E	del exon 45	DMD	Ambulant at sampling	9yrs
F	del exon 45	DMD	Ambulant at sampling	6yrs
G	del exon 45	DMD	Ambulant at sampling	7yrs
H	del exon 45	DMD	Ambulant at sampling	6yrs
I	del exon 45-50	DMD	Ambulant at sampling	10yrs
L	del exon 45-50	DMD	Ambulant at sampling	12yrs
M	del exon 45-50	DMD	Ambulant at sampling	7yrs
N	del exon 49-50	DMD	Ambulant at sampling	7yrs
O	del 50	DMD	Ambulant at sampling	10yrs
P	dup exons 65-79	DMD	Ambulant at sampling	19yrs
Q	c.4117c>T, p.Q1373X	DMD	Ambulant at sampling	6yrs
R	c.9204-9207del,p.N3068K,fs*20	DMD	Ambulant at sampling	8yrs

Table 4 - Selected patients for Csnk1ε protein quantification in plasma. Mutation, phenotype and age at sampling are listed.

3.4.5. Csnk1e specific ELISA assay

ELISA assay was performed using CSNK1ε ELISA kit (MyBioSource) according to the manufacturer's instructions. In brief, a total of 100 µl of standard and sample were added to a pre-coated microplate (96 wells) with antibody specific for Csnk1ε and incubated for 2 hours at 37ºC. Each sample was tested in triplicate. Following that, the liquid was discarded without washing, and a 100 µl of Biotin-antibody (1x) was added to each well and incubated for 1h at 37ºC. Each well was washed for 2 min using 200 µl of wash buffer

for a total of three washes. Then 100 µl of HRP-avidin (1x) was added to each well and incubated for 1h at 37ºC and then washed five times with the washing buffer. After that 90 µl of TMB substrate were added and incubated for 15 min at 37ºC. Finally, 50 µl of stop solution was added and the optical density of each well was determined using a microplate reader at 450 nm within 5 minutes.

For data analysis, standard curve has been constructed using "Curve expert 1.3" software, according to the manufacturer's instructions. Briefly, mean absorbance for each standard was plotted in the x-axis against the concentration on the y-axis and the best fit line was drawn through the points on the graph.

For each sample, three absorbance measurements were done and averaged. Csnk1ε concentration had been calculated based on absorbance value in relation to calculated standard curve according to the following equation: [Csnk1ε]= (mean absorbance - 0,0391) / 0,0013 and finally expressed in pg/ml .

For all samples Csnk1e plasma concentration has been studied considering age at sampling, mutation type and clinical severity (see below).

4. RESULTS

4.1. Circadian genes are deregulated in mdx mouse model

4.1.1. Mdx sedentary vs wt

In order to explore the involvement of circadian genes in dystrophinopathies, we designed *ad hoc* a low density micro fluidic-card TaqMan based assay (LDTA), Fluid-CIRC, to obtain the transcriptional profiling of two different mdx muscles, gastrocnemius (GC) and tibialis anterior (TA), from at least 3 different animals. Mice were both sedentary and trained with a specific schedule of exercise (see above in Table 1). These muscles had been selected because of their involvement in mdx pathology and, in particular for GC, for its muscle fiber composition (both fast and slow fibers are represented) and its large functional involvement in horizontal treadmill exercise as applied in our study (Camerino et al. 2014).

GC and TA muscles from 3 different mdx each, were compared with as much as *wt* muscles, age matched (8-12 weeks for GC and 16-20 weeks for TA) for the differential expression of selected genes (Tab.1).

As shown in Figure 7A in gastrocnemius muscle there is a profound deregulation of all core clock genes as well as MyoD and MyoG. In particular an evident downregulation affects 90% of selected genes except for MyoG, Tim, and Tgfb1 that are upregulated, with the first (MyoG) as the most expressed one.

The same trend is present in tibialis anterior of the same cohort of mdx sedentary versus *wt*: 96% of analyzed genes are markedly down-regulated but, as for gastrocnemius, MyoG is greatly upregulated (Figure 7B).

Timless (Tim) up-regulation was relevant only in TA muscle and this behavior could find an explanation considering the Per1 and 2 downregulation as this gene is part of the negative loop of inhibition of Clock/Arntl1 activated Per proteins.

In any case, what is particularly relevant, and more evident in GC, is the loss of a mutual variability of expression among Core Clock Genes (CCGs) as might be expected in a regulatory network based on feed-back loops.

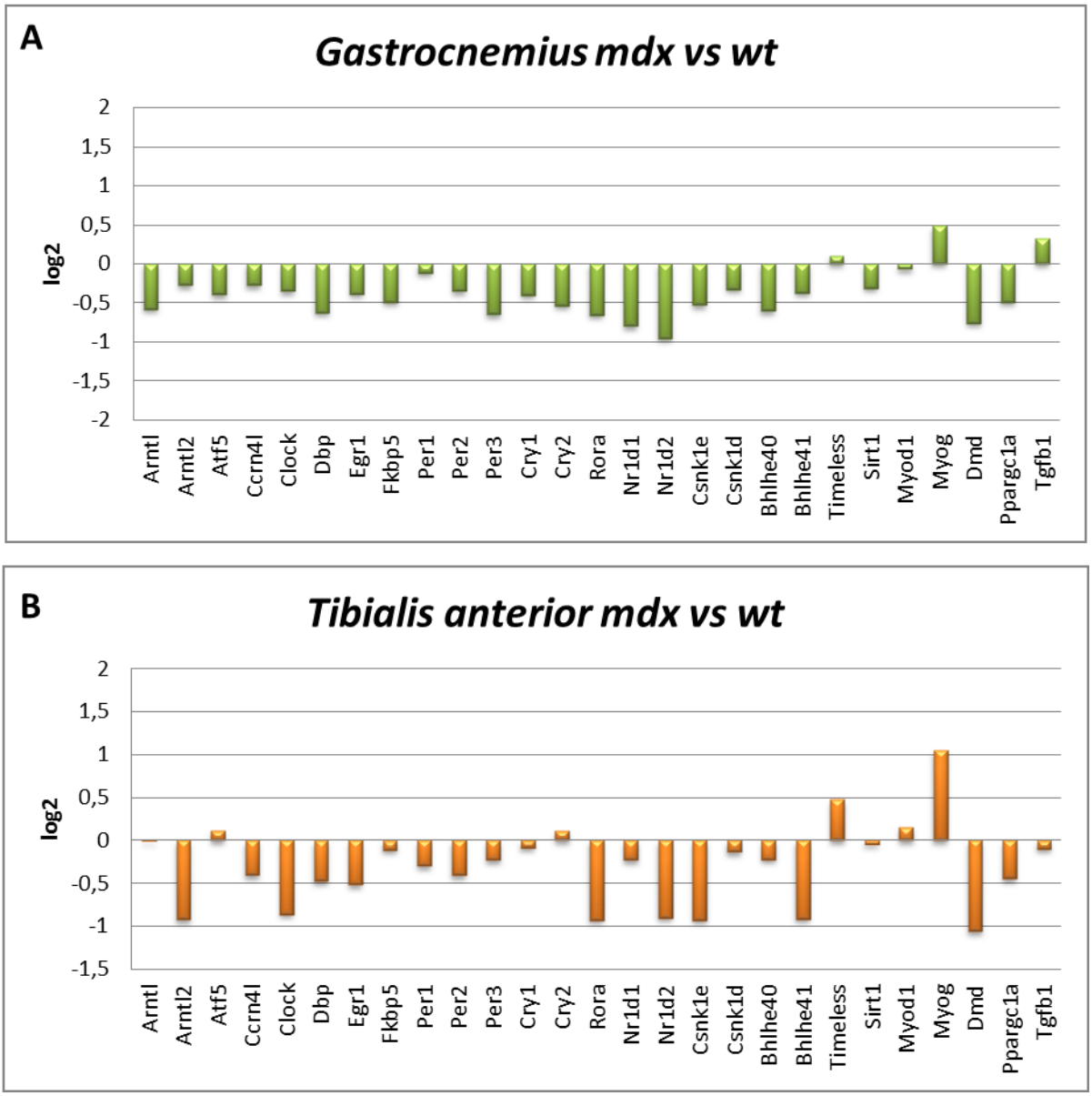

Figure 7 - Fluid-CIRC expression data of GC (A) and TA (B) mdx respect to wt. All CCGs are strongly deregulated with a trend through a general down regulation. Only MyoG is consistently up-regulated in both muscle types.

4.1.2. Mdx exercised vs mdx sedentary

The same comparative analysis was conducted for exercised mdx with respect to sedentary mdx. As explained before, specific scheduling of exercise can have a damaging effect on muscle of the mild mdx phenotype contributing to create a more reliable model of Duchenne disease. For our purpose a total of 6 muscles from exercised mdx, 3 for GC and 3 for TA were compared with muscles from age matched mdx mice.

Comparing mdx-exercised cohorts with mdx sedentary ones we can observe an almost opposite trend of gastrocnemius with respect to the tibialis anterior (Figure 8), and in

particular for GC, exercise seems to completely change the expression side of all genes respect to untrained mdx.

While in GC muscle 100% of genes are strongly up-regulated with the only exception of Egr1, on the contrary the tibialis anterior's genes expression trend seems to be opposite with respect to gastrocnemius. This peculiar behavior could possibly be due to the different fibers' type composition of the two muscles and the consequent response to the exercise schedule. In any case, in both muscle types all the core clock genes are deregulated in response to exercise with respect to mdx mice sedentary, and of note is the great upregulation of core clock genes and DMD in gastrocnemius. Interestingly, MyoG and MyoD don't show a sensible change in expression respect to mdx mice in both muscle types (Figure 6).

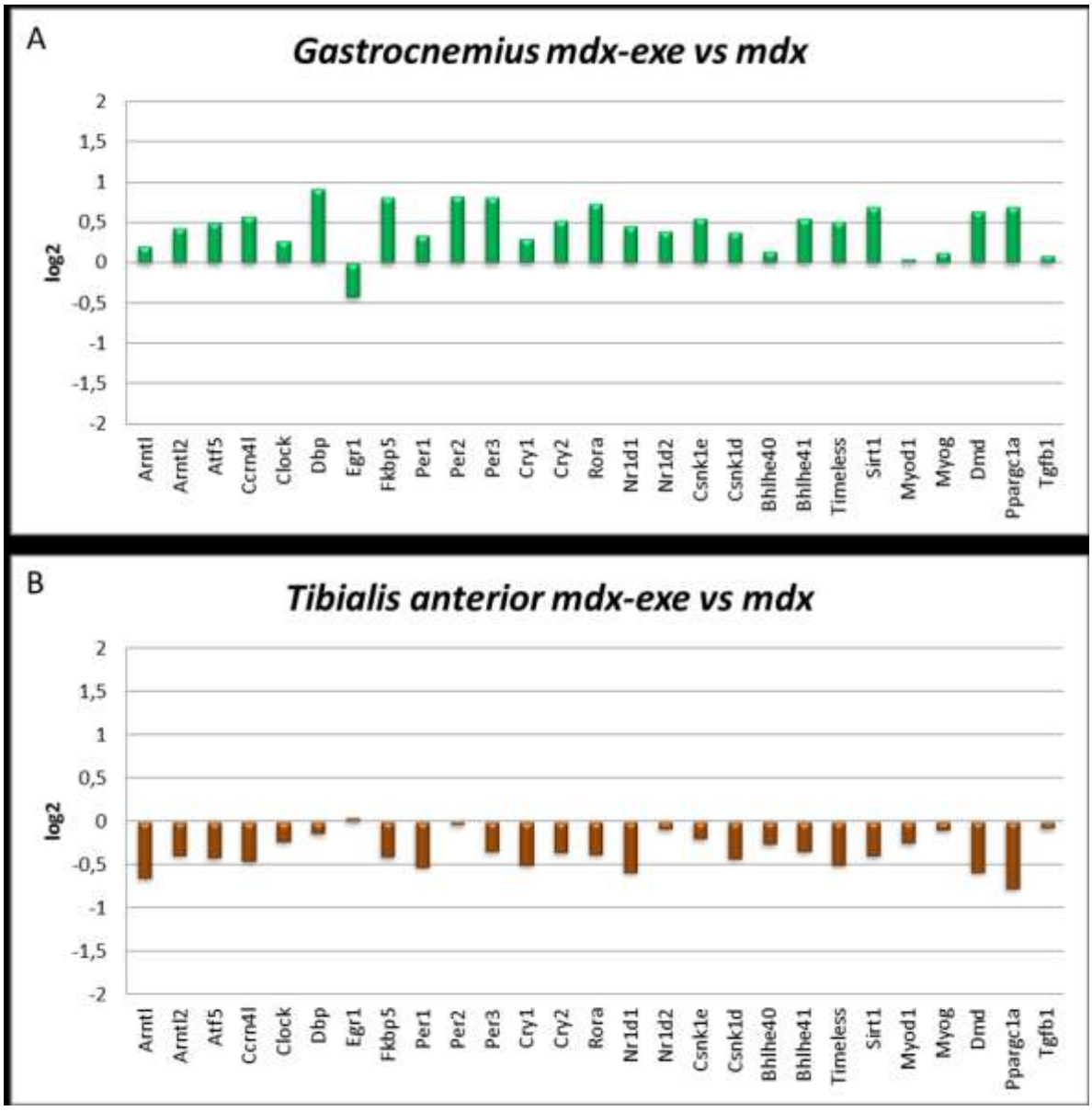

Figure 8 - Fluid-CIRC expression data of GC (A) and TA (B) of mdx-exe vs wt. All CCGs are strongly deregulated. Note the opposite behavior of the two selected muscle types. MyoG and MyoD don't show a consistent change of expression respect to mdx.

4.2. 7 most deregulated genes prioritization

Using an algorithm for statistical analysis set up by Elena Schwartz (Ariadne Genomics, Rockville MD, USA) starting from Fluid-CIRC expression data, we prioritized seven of the most deregulated genes according to their change in expression degree and the key role in relevant pathways both in DMD and in circadian circuitry. The selected genes were Arntl, Cry1, Cry2, Csnk1e, Sirt1, MyoG, MyoD. The proposed genes were selected because their expression was the most consistent in all samples and they have the biggest changes. MyoG is really downstream of MyoD, which is also confirmed by the similar

patterns of expression. The biological role of deregulated circadian genes is explained in Figure 9 where the interaction pattern is depicted: Cry1/2 and Arntl1/2 go up because Sirt1 and Csnk1ε go down as predicted by the circadian clock regulation pathway.

In Figure 10 the pathway of interaction of the 7 deregulated genes is reported: Arntl, Cry1 and 2 are the main components of the core clock circuit and Csnk1ε is a downstream regulator involved in post-translational phosphorylation of key effectors. Sirt1, a NAD+ dependent histone deacetylase, binds Clock-Arntl1 heterodimer and promotes Per2 deacetylation and degradation. In muscle tissues, it is part of the AMPK/Sirt1/Pgc1α pathway a pivotal regulator of fast to slow fiber switch in response to stress and exercise. MyoG and MyoD, as they are known, are bHLH transcription factors involved in muscle regeneration and differentiation; they are under direct control of the Clock/Arntl1 heterodimer and their deregulation could possibly represent the key node of conjunction between circadian deregulation and muscle pathology.

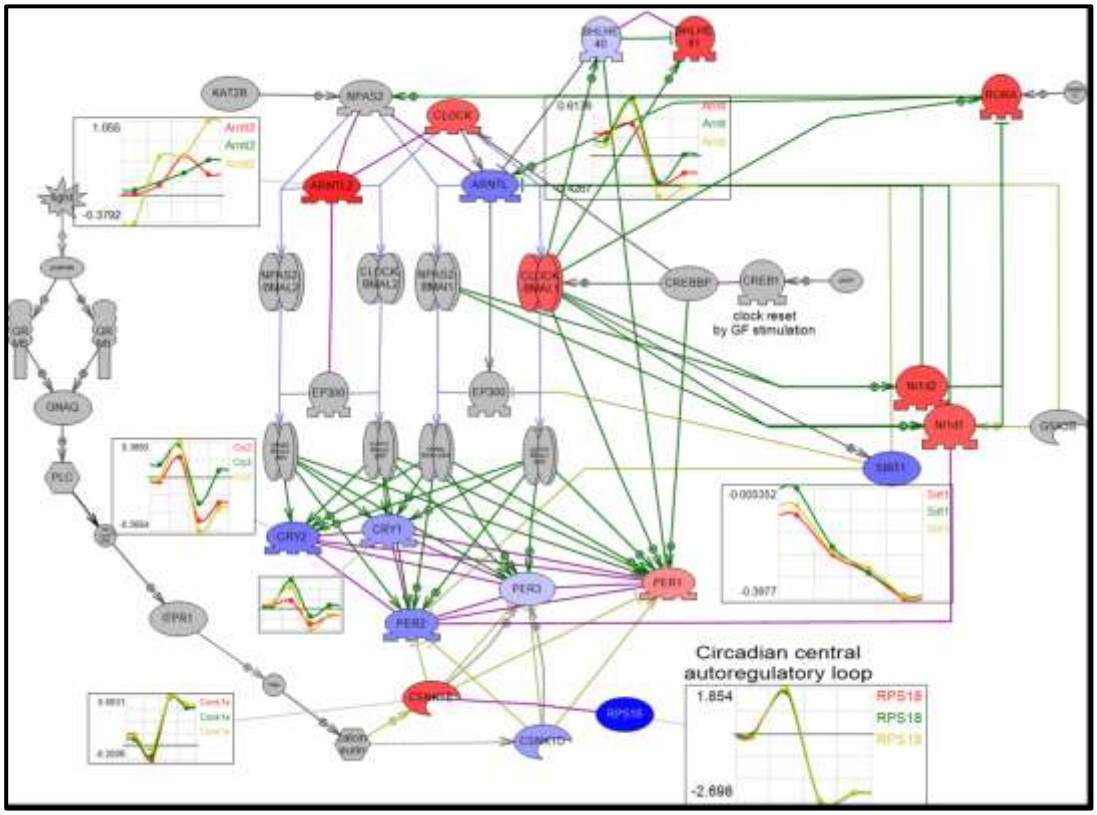

Figure 9 - Biological role of deregulated circadian genes: Cry1/2 and Arntl1/2 go up because Sirt1 and Csnk1ε go down as predicted by circadian clock regulation pathway.

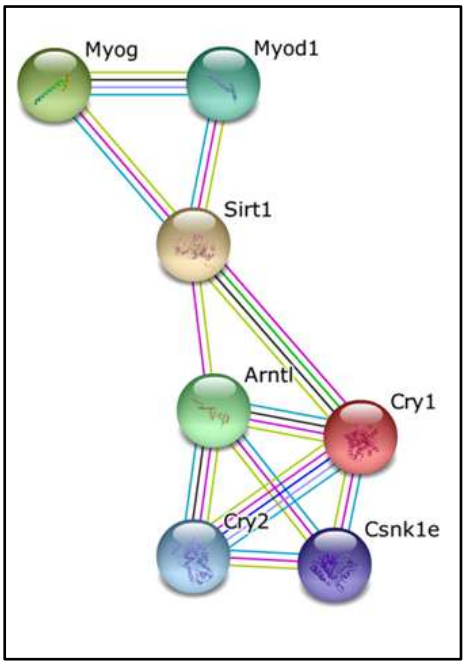

Figure 10 - Interaction pathway (SPRING) of the 7 most deregulated genes.

4.3. Different drug treatments improve circadian genes deregulation in mdx mice

In order to explore a possible positive effect of different drugs currently under broad experimental evaluation as possible treatments for DMD, we analyzed 20 mdx mice treated with 7 different compounds: prednisolone, apocynin, taurine, resveratrol, pentoxifylline, enalapril and nandrolone. For each treatment 3 GC samples were run in our custom Fluid-CIRC and data for the 7 most deregulated selected genes were analyzed according to the $2^{-\Delta\Delta Ct}$ method; for enalapril only 2 GC samples were available and analyzed.

These compounds were chosen because of their mechanism of action and the demonstrated effect in ameliorating DMD muscle pathology. Prednisolone is a steroid compound widely used in DMD for its undoubted positive effect in delaying loss of ambulation time, and improving cardiac and respiratory performance and has an anti-inflammatory effect; apocynin is a NOX2 inhibitor and reduces oxidative stress and NF-kB activation in exercised mdx, via angiotensin II (Capogrosso et al. 2016); taurine is a natural amino acid normally present in muscle, where it controls calcium homeostasis and excitation–contraction coupling; resveratrol is an anti-oxidant and anti-inflammatory compound that acts in promoting fast to slow switching and mitochondrial metabolism via AMPK/Sirt1/Pgc1α pathway; pentoxifylline is a phosphodiesterase inhibitor that ameliorates calcium homeostasis and regeneration increasing cytoplasmatic AMPc levels; enalapril is an angiotensin II inhibitor already widely used for its positive role in improving

cardiac function and preventing late heart and muscle fibrosis; finally nandrolone is an anabolic drug studied for its effects in improving muscle mass and consequently muscle force.

In Figure 11 gene expression data of muscle samples for each treatment are depicted: a general trend of down regulation toward a normalization of the altered expression respect to wild type is visible in all. The up-regulation of Cry2 could probably be explained as part of the normal circadian circuitry: Cry proteins are part of the negative feedback loop, and they inhibit Clock/Arntl1 heterodimer, and indeed Arntl1 is down regulated in all treatments except for resveratrol. This interesting data could possibly suggest a positive effect of the treatments in ameliorating general muscle pathology reverting circadian circuitry deregulation to a more "normally" oscillating one.

The most interesting data is the complete reverted upregulation of MyoG in all treatments. This finding could be interpreted in a double fashion: considering that MyoG is under the direct control of Clock/Arntl heterodimer, it could be down regulated because of the reduced expression of Arntl as above mentioned, conversely, MyoG transcript reduction could be considered a direct effect of the different treatments, influencing muscle pathology.

In resveratrol treated samples the general trend described for the other drugs is not represented and a general up-regulation of all circadian genes together with Sirt1 could be shown, this latter effect being expected to be based on the known pharmacological effects of the compound in stimulating AMPK/Sirt1/Pgc1α pathway.

All together these data demonstrate that drug treatments, already known for their positive effect in DMD pathology, are able to normalize a deregulated expression profile of both circadian genes, MyoG and Sirt1, supporting the hypothesis of these genes as being possible good candidate biomarkers of disease severity.

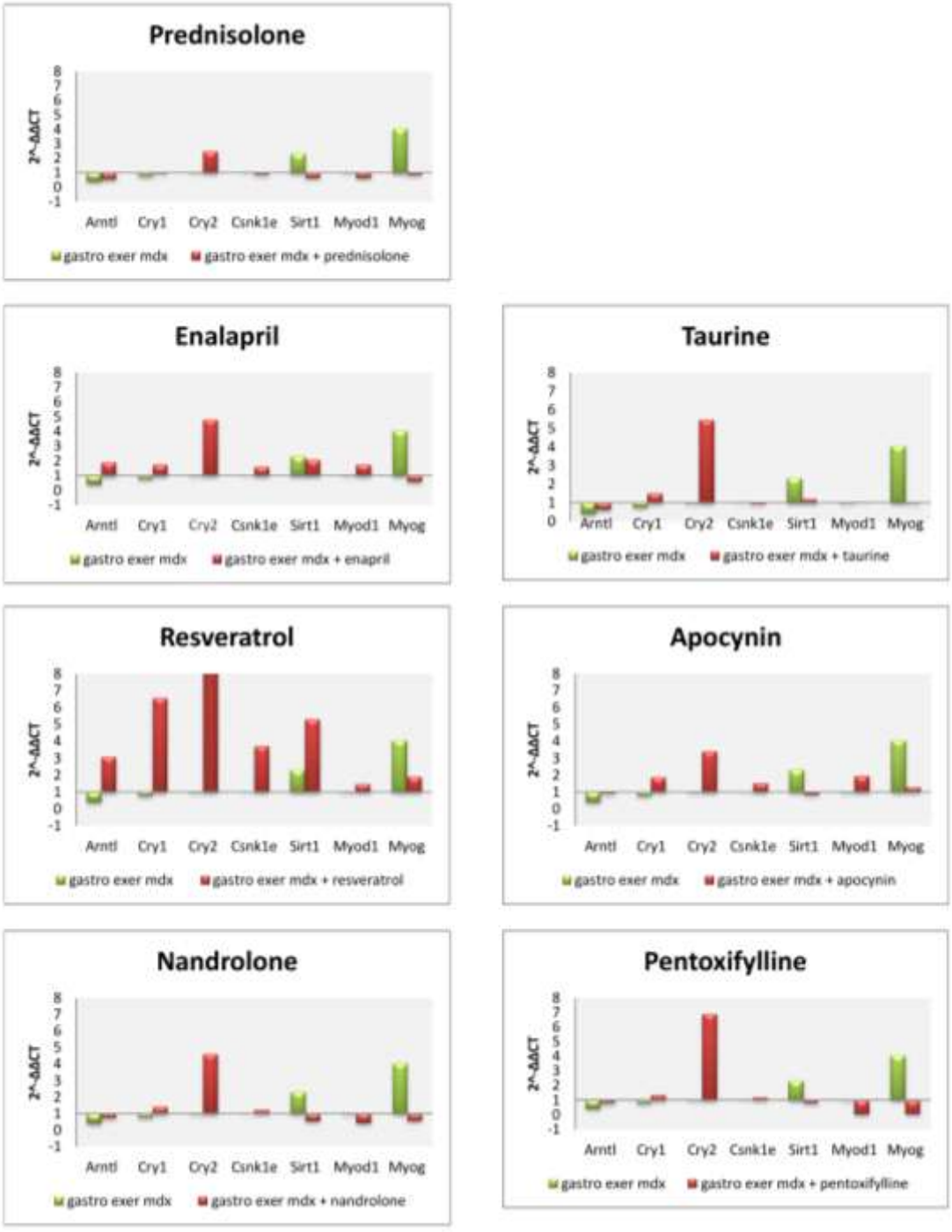

Figure 11 - expression profiling of the 7 deregulated selected genes in GC after treatment with different drugs with known positive effect on Duchenne muscular dystrophy.

4.4. Prioritized circadian genes, MyoG and Sirt1 are deregulated in DMD patients

Considering results obtained in mice studies, we decided to explore the expression profile of the seven deregulated genes in muscle biopsies from DMD patients. 10 subjects with different mutation types were selected because of the exploratory meaning of our research.

Interestingly all analyzed muscles showed the same deregulation as observed in animal studies. A part from expression levels varying among different samples, possibly due to muscle quality, muscle fiber composition, disease stage, a general up-regulation of Cry proteins was clearly visible and reflects the down regulation trend seen for Arntl as part of the negative feed-back loop as already described (Figure 12).

Nevertheless really high expression levels were evident in particular for Csnk1ε, Sirt1 and MyoG in almost all samples, with the only exception of PT8 and PT10, but considering the very low levels of all the transcripts, this observation can be related to samples' quali/quantitative variability.

Among the three, MyoG is greatly upregulated in all samples, with PT5, PT6 and PT9 reaching levels up to more than 30 times with respect to *wt* (Figure 12)*;* MyoD doesn't reach such high levels suggesting a possible independent role of MyoG in disease pathology as part of a specific altered pathway. To our knowledge this is the first time in which MyoG expression has been specifically explored in muscles from DMD patients. Its upregulation together with Cnsk1ε and Sirt1 as the most upregulated among the 7 selected genes, could be of particular interest to be further explored as candidate biomarkers in human patients.

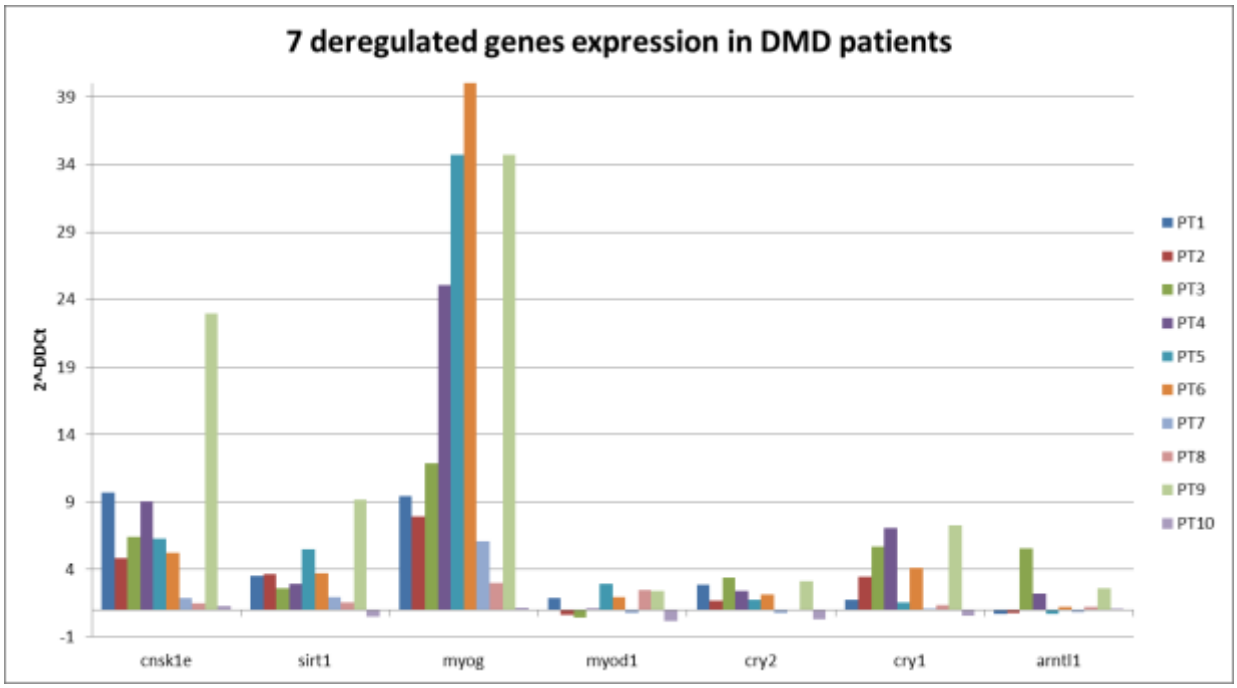

Figure 12 - Expression profiling of 7 deregulated genes in muscle samples from DMD patients. Note the great up-regulation of MyoG.

4.5. Cnsk1ε plasma levels are slightly elevated in Duchenne patients

Expression data in patients, and in particular upregulation of MyoG, primarily and then Cnsk1ε and Sirt1, together with the absence of specific studies in DMD patients in literature, prompted us to further explore if these specific transcripts deregulation could reflect in plasma of patients affected by dystrophinopathies. At the moment of drafting the present thesis only data about Cnsk1ε are available and will be described.

For proteomic studies plasma samples from a total of 16 patients were obtained, 14 DMD, 2 BMD, as well as 5 controls were selected and analyzed with Cnsk1ε specific ELISA kit (MyBioSource). All DMD patients were ambulant at sampling and homogeneous for age (around 9-10 years on average), except for patient P who was 19 yrs old.

BMD patients were less homogeneous, as patients A loose ambulation at age 20 yrs, on the contrary patient B was still ambulant but was really young (4 yrs).

To draw standard curve, mean absorbance for each standard was plotted in the x-axis against the concentration on the y-axis and the best-fit line drawn through the points on the graph (Figure 13).

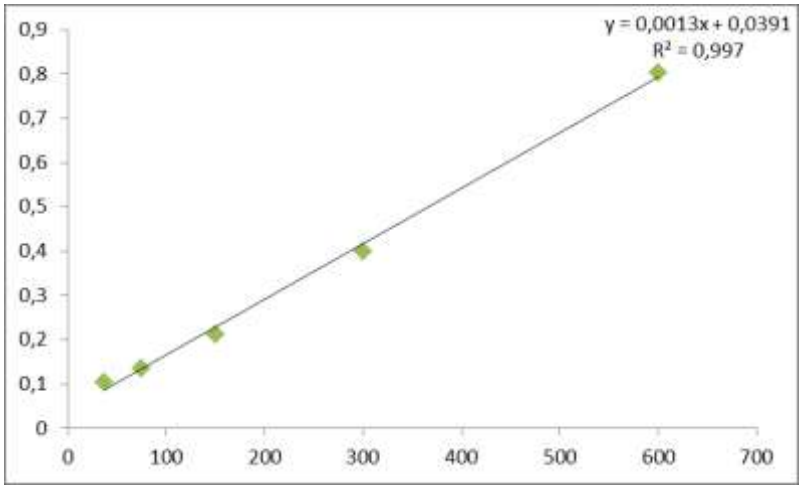

Figure 13 - Standard curve for Csnk1ε specific ELISA assay.

Cnsk1ε protein concentration is depicted in Figure 14. Protein concentrations as measured in the 5 controls were averaged and used for the comparison.

Although variable, in general all samples demonstrated slightly elevated plasma levels with respect to controls, a part from G, H, I DMD patients which have about 2 times higher Cnsk1ε plasma levels.

In the attempt to find a possible correlation with plasma protein levels and different variables, data were analyzed based on phenotype (DMD or BMD) and mutation type: unfortunately no clear correlation could be found. Notably, Cnsk1ε higher levels were seen in two patients with deletion of exon 45 one of the most frequent mutations localized in one of the well-known mutational hot spot of the dystrophin gene.

To verify a possible role of this protein as a marker of disease severity, plasma concentrations were also evaluated based on age at plasma sampling, but also in this case we didn't find any specific correlation trend with respect to age and therefore disease worsening.

However, the sample's cohort analyzed for both BMD and DMD patients and healthy controls, proves to be rather limited and an enlargement of the number of the samples could lead to a better definition of Csnk1ε protein profile.

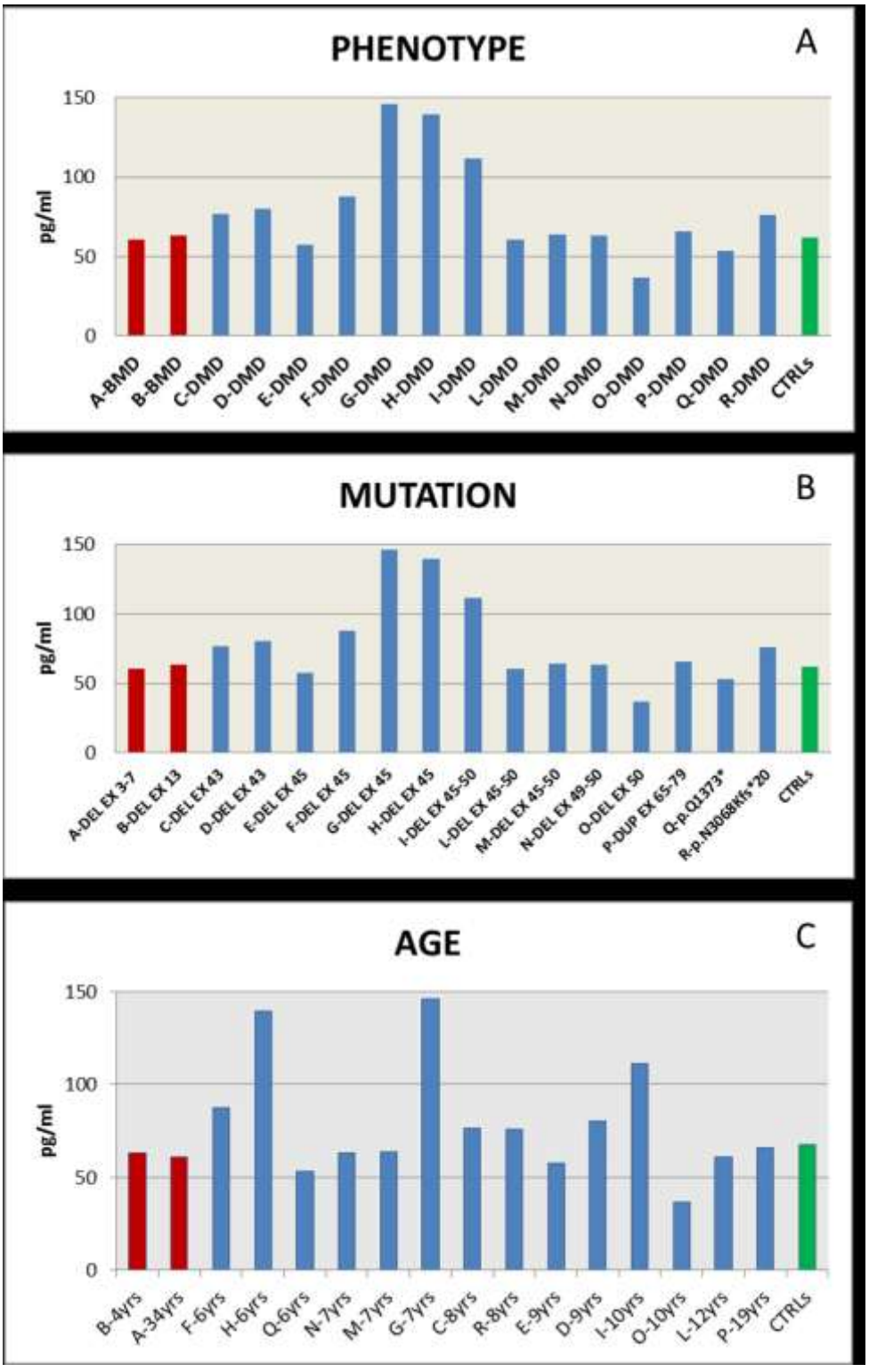

Figure 14 - Csnk1ε protein plasma levels in DMD patients analyzed with respect to phenotype (A), mutation type (B) and age (C). Green bars are averaged controls, red bars are BMD patients, blue bars are DMD patients.

5. DISCUSSION

Duchenne muscular dystrophy is a severe muscle wasting hereditary disease for which no cures are available. Many different strategies and drug compounds are under active research, some of which are already in clinical trials. Although there are relevant recent advances in that field, many problems still remain to be overcome: many drugs have demonstrated efficacy but low efficiency (es. antisense oligonucleotide based therapy or translarna), trials design is challenging due to low numbers of patients available, outcome measures still lack precision and reliability to even minimally highlight positive responses to innovative treatments and finally toxicity sometimes hinders the passage to following steps in the drug development clinical process.

To overcome these problems biomarkers have attracted a great interest in recent years. As already mentioned they are parameters, that can be reliably measured and can give direct information about different characteristics of a given pathology (Scotton et al. 2014). In these terms diagnostic, prognostic and therapeutic biomarkers have been delineated.

Considering DMD, biomarkers could avoid invasive procedures for diagnosis (muscle biopsy), may facilitate follow-up and patient stratification based on prognosis. This latter aspect is really important in subject selection for clinical trials. In addition the availability of therapeutic as well as pharmacogenomic biomarkers can give easy accessible and more reliable outcome measures for efficacy evaluation of innovative treatments.

Recently Scotton and colleagues (2016) have demonstrated a profound deregulation of circadian rhythm genes in collagen VI related myopathies, paving the way to the discovery and validation of new biomarkers in rare neuromuscular disease.

With these premises, we decided to study the possible involvement of circadian rhythm also in the more frequent Duchenne muscular dystrophy.

We started studying expression profiling of selected circadian genes together with some gene involved mainly in muscle function and regenerative process in mdx mouse model. For this purpose we designed an *ad hoc* custom micro fluidic card TaqMan based assay called Fluid-CIRC. This tool has demonstrated to be highly effective and time consuming allowing a complete and accurate transcriptional profiling of 32 selected genes in GC and TA muscles of both *wt* and mdx mice, sedentary, exercised and treated with different drugs.

Nowadays animal studies have regained importance because, although expensive and high demanding in terms of time and the setting up of experiments, they allow for taking steps forward in the human application for more promising results.

The mdx mouse model is the widely used model of DMD although affected by a less severe phenotype. For this reason we decided also to explore a possible circadian deregulation in the more severely affected exercised mdx as recently described by Camerino et al. 2014.

Interestingly our animal studies revealed a profound deregulation of all circadian genes together with muscle regeneration marks such as MyoD and MyoG. In particular down-regulation of all analyzed genes was the general trend in both GC and TA except for MyoG and TGFb1 showing a relevant up-regulation. Exercise demonstrated to worsen this trend and although in TA muscle the way of deregulation didn't change, on the contrary in GC an opposite direction of expression was evident. This data is not a surprise considering the different fiber type composition between the selected muscles and the mechanic function of GC, more involved by the exercise schedule used in the present work. Furthermore, a different behavior of circadian clock genes in different tissues and muscle types, also in the same animal, is a well-known phenomenon, underlying the need to further investigate the behavior also of single muscle types or functional groups (Wolff et al. 2012).

MyoD and MyoG upregulation in mdx was already reported (Onofre-Oliveira et al. 2012) as the signature of satellite cells activation and differentiation stimulus. Also Tgfb1 upregulation is a known pathological sign of DMD reflecting the activation of end stage pathways mainly involved in the final fibrotic muscle degeneration (Dadgar et al. 2014).

These promising data prompted us to continue exploring circadian genes involvement in DMD. For this aim and with the intent to finally reach the goal of exploring them in human patients, we decided to narrow down the number of genes prioritizing only the most deregulated ones, in order to obtain more robust disease hallmark, the role of which is to be further elucidated. Collaboration with Elena Schwartz from Ariadne Genomics within the EU project BIO-NMD (www.bio-nmd.eu) helped us to select 7 most deregulated genes applying various statistic algorithm based on Fluid-CIRC expression data. As described initially in Kotelnikova et al. this strategy ended up being truly helpful to identify only key regulators crucial for the pathways studied and consistently deregulated. Thank to this approach based on in silico tools, biomarker discovery procedures could be speeded up and focalized only in really promising molecules. This is a crucial point in biomarkers

discovery workflow, in particular in a high throughput era in which a huge amount of data are obtained in a relatively time and cost consuming manner. The seven identified most deregulated genes were: Arntl1, Cry1 and 2, Cnsk1ε, Myod, Myog and Sirt1. The first four are Core Clock Genes involved in the principal feed-back loop of circadian cellular regulatory pathway. Arntl1 forms a heterodimer with Clock, which translocates into the nucleus binding different E-box elements of as many as different genes promoting their transcription. Its deregulation has important consequences on muscle health. First Arntl1$^{-/-}$ mouse shows a decrement in force generating capacity, sarcomere and myofilament structural alterations and mitochondrial pathology such as number reduction and mitochondria swelling (Andrews et al. 2010). Secondly, the heterodimer Clock/Arntl1 directly regulates MyoD and disruption of this latter leads to impaired regeneration. Recently, Dadgar and colleagues have demonstrated that the asynchrony of regenerative signals may cause the failure of the process itself and that this impaired mechanism could be at the base of Duchenne pathogenesis as well as for other muscular dystrophies. In this perspective, circadian gene deregulation may affect MyoD expression inducing an alteration in circadian equilibrium of muscle cell activities driving pathology.

Cry 1 and Cry 2 together with Cnsk1ε are part of the same circuitry: casein kinase phosphorylates Per proteins that are linked to Cry ones forming the inhibitory loop that inactivate Arntl/Bmal. In particular Cnsk1ε has recently given rise to interest because of its implication in many degenerative diseases, such as Alzheimer as well as cancer (Bibian et al. 2013). Indeed Casein kinase family proteins regulate many cell processes including not only circadian rhythm but also signaling, cell transport, DNA repair. In Alzheimer disease a constitutive activation of this proteins seems to promote amyloid precursor protein APP production and deposition. Altered expression levels of casein kinase ε have been associated with cancer onset and prognosis due to its involvement in cellular proliferation, in breast and colon-rectal cancer, as well as hepatocellular carcinoma (Lopez-Guerra et al. 2015; Richter et al. 2015; Lin et al. 2016). Interestingly, highly selective casein kinase inhibitor for anti-proliferative therapeutic purposes are under drug development (Bibian et al. 2013).

Regarding Sirt1, it is a NAD^+ dependent histone deacetylase and regulates the expression of many genes modulating histone acetylation state. Among its target both MyoD and Pgc1α could be found: the first, as already described, has a pivotal role in muscle development and regeneration, the second is a key protein in promoting the slow oxidative

program activation. Sirt1 expression level may have fluctuations in particular in stressful conditions such as starvation, muscle contraction and regeneration. Recently, Amat et al. (2009) demonstrate that Sirt1 promotes Pgc1α activation, and that Myod facilitate this interaction contributing to the formation of a complex with the ability to drive change in gene expression relative to skeletal muscle cell requirements.

Finally, in mice, MyoG demonstrated to be the most deregulated, in particular in GC muscle. Myogenin is part of the HLH muscle specific transcription factors together with MyoD, Myf5 and MRF4. It stimulates muscle differentiation (Meadows et al. 2011), is mainly expressed in slow oxidative fibers, and if overexpressed it promotes the fast to slow switch process (Hughes et al. 1999). It has recently been reported in a study whereby the authors created a mdx mice MyoG-deleted: this animal didn't show any specific histologic alteration and no impairment in the muscle regeneration process, demonstrating that MyoG is dispensable for this latter and doesn't affect muscle degeneration. Nevertheless, MyoG deleted mdx mice demonstrated to be able to run for a greater distance with respect to mdx, approaching *wt* animals, and it seems to have an effect on muscle metabolism, in particular enhancing nNOS expression, explaining the better performance and the incremented threshold to fatigability demonstrated by this animals (Meadows et al. 2011). All together these data supports the hypothesis of a positive effect of MyoG downregulation in dystrophic muscle.

In mdx treated mice, expression analysis surprisingly demonstrated a general trend toward the normalization of deregulated gene expression levels for all drug tested. Among all, MyoG down regulation was the most evident. Based on Meadows et al. publication, this data seems to be really promising suggesting MyoG as a possible candidate as prognostic and therapeutic biomarker to be further analyzed. Indeed a myogenin up-regulation in pathologic tissues could be interpreted as a clear sign of activation of muscle specific pathways in order to react to contraction damage due to dystrophin absence. Regeneration based on satellite cells activation is the first step and in this context MyoG acts in the final differentiation process allowing myotubes final fusion and adult muscle maintenance. Furthermore, from Meadows et al. (2011) we know also that MyoG stimulates the fast to slow fibers switch program largely used by muscle cells because it represent a phenotype more useful to contrast degenerative process. In these terms, finding such a great downregulation of MyoG in response to almost all drug treatment tested is a really

promising data, suggesting also a pivotal role of this transcriptional factor in DMD pathogenesis.

In clinical trials for DMD, one of the most widely used physical outcome measure to evaluate drug efficacy is the "six minutes walking distance test" (6MWD). It consists in asking patients to walk for a fixed distance of 25 m up and down for 6 minutes: the final meters covered are registered and used to infer physical performance of the patient. Evaluation of the effect of the ongoing innovative treatments are mainly based on the use of this outcome clinical parameter. Considering that and based on previous results showing an improvement in distance run in mdx MyoG deleted mice, analyzing a possible correlation between MyoG mRNA tissues expression or plasma protein levels respect to physical performance in patients, maybe in terms of 6MWD test, could give the base for the development of a possible therapeutic biomarker.

Finally, as mentioned, MyoG together with MyoD are under direct control of Clock/Arntl1 heterodimer, so MyoG might represent the link in between.

Resveratrol treatment deserves to be mentioned: in this case a general upregulation of all the 7 selected genes was evident, a part from MyoG, and Sirt1 expression was really enhanced together with Cry proteins. This peculiar behavior was not surprising because the positive effects of resveratrol in stimulating AMPK/Sirt1/Pgc1α is clearly known and there is a rise of great interest for its possible therapeutic application in Duchenne muscle dystrophy together with other molecules able to enhance AMPK pathway stimulation (reviewed in Ljubicic et al. 2017).

Such promising results in the mdx animal model prompted us to proceed with our research on human subject: expression profiling of selected genes was carried out in 10 DMD patients with the intent to highlight the same gene expression deregulation. Completely in accordance with animal studies, gene expression in muscle from selected patients demonstrated a great deregulation, and in particular MyoG, Sirt1 and Cnsk1ε were the most significantly and consistently upregulated in all samples.

In order to finally explore a possible reflection of this deregulated behavior also in protein product, an ELISA immunoassay was used to investigate MyoG, Sirt1 and Cnsk1ε levels in plasma of 16 patients, 14 DMD and 2 BMD. This is the first time in which these proteins have been investigated in plasma. As mentioned, Cnsk1ε has recently attracted attention as biomarker in cancer, but it was only explored in terms of expression in neoplastic tissues; the same for MyoG: together with Myod it has already been explored as

a diagnostic biomarker in pediatric rhabdmyosarcoma but only in terms of transcript levels in cancer tissues (bone marrow) (Michelagnoli et al. 2003). Sirt1 expression is widely investigated in muscle tissue because of its known involvement in fast to slow fiber switch program activation, but as far as we know, it has never been explored as a possible biomarker to be measured in plasma.

At the moment of drafting the present work, ELISA assay for MyoG and Sirt1 protein quantification in plasma of affected patients are in progress.

Regarding Cnsk1ε data, although plasma levels seem not to be hugely affected and no clear correlation could be drawn with respect to age, phenotype and genotype, nevertheless a trend toward a general elevation could be inferred.

A larger patient cohort is needed in order to better define a possible role of this kinase as a disease biomarker, in particular considering recent advances in specific inhibitory drugs development, that could finally be useful also in DMD.

In any case, all together these data demonstrate circadian rhythm deregulation in Duchenne muscular dystrophy, paving the way for a new research field that deserve to be further explored for its diagnostic, prognostic and therapeutic implications not only in DMD but also in other degenerative neuromuscular diseases.

5.1. Circadian rhythm genes and DMD: reflections

The discovery of the involvement of circadian rhythm in the pathogenesis of collagen VI myopathies provided authors the problem of how to explain a possible correlation between circadian circuit and muscle pathology driven by mutations in a protein belonging to extracellular matrix environment.

This is the same problem we face at the end of this work. Because, if it is true that there is a great body of evidence regarding the relevance of circadian rhythmicity and skeletal muscle health, it still remains to be explained what the real role of core clock genes disruption in the pathogenesis of the disease.

Both hypothesis as described in Scotton et al. 2016 could find the same rational: circadian disruption in DMD could be a secondary downstream effect of the disease, or on the contrary an independent variable acting as a possible disease modifier.

Dystrophin absence has a great impact on muscle health and starting from the failure of exerting its role as mechanotransducer during muscle contraction, membrane damage gives rise to a cascade of downstream pathways activation many of which normally acting in

muscle cells but disrupted in terms of timing and quali/quantitative expression. As in many other diseases, in the attempt to react positively and adapt to a negative situation cells activate at multiple levels and within successive waves due to the continuous degenerating stimulus contraction induced (Dadgar et la.2015).

At the same time circadian circuitry as well as all downstream effectors, among which MyoG, Sirt1 and Cnsk1ε resides, normally work in a delicate molecular feedback balance promoting muscle health.

In this way, the great and multilevel genes expression deregulation could disrupt the delicate balance between main actors such as fast to slow switch metabolic key regulators (Sirt1), muscle differentiation (MyoG), and the time in which all these adaptive changes have to occur. Considering our results, such a great up-regulation of MyoG, which together with MyoD is under the direct control of Clock/Arntl1 heterodimer, could alter the delicate equilibrium of transcriptional/translational feedback, and this could reflect in all CCGs regulation upstream.

In these terms, circadian circuit involvement in Duchenne muscular dystrophy may be a consequence of the disease, worsening its course and impacting in drug treatment outcomes, and deserves further studies. Firstly because among CCGs good prognostic and therapeutic biomarkers could be found, but also by elucidating its role there could be relevant implications in terms of drug discovery and chronotherapy. Compounds directed to re-establish circadian rhythm disruption in skeletal muscle could be valuable adjuvant for other more specific, defect-oriented approaches. Furthermore, considering the well-known circadian response to external stimuli made by skeletal muscle, and its relevance in improving performance for example in athletes, specific schedules of drug administration as well as physical therapy could better improve disease outcome with greater implication in terms of personalized treatment: give the correct therapeutic intervention to the most responsive patient at the best time to reach the better response.

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