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Urine-derived stem cells are a novel approach for transcriptomic studies in Duchenne Muscular Dystrophy

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DEDICATION

"Many of life's failures are people who did not realize how close they were to success when they gave up" Thomas A. Edison

To My Wonderful Parents

My Mother Nohma and my Father Mohammad Bassam

Thank you, from the bottom of my heart for supporting me until the end, for making me believe that nothing is impossible in this life. Without your thoughtful prayers and continuous encouragement, I would not have been able to reach this goal.

To my amazing sisters Lina and Mariam

Thank you, for always being there for me even though we are physically apart.

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ABSTRACT

Background: Nowadays, urine derived stem cells (USCs) are considered as a new approach for modelling a wide variety of human diseases. In fact, these cell lines have many advantages in terms of the easy isolation, low costs and the absence of ethical issues.

Aims: The goals of this study included examining the gene expression profile of both native and myogenically transformed (MyoD) USCs derived from DMD patients and healthy individuals. In addition to that, we exploited the new technology Celector ® (StemSel Ltd.), in order to distinguish both control and DMD USCs through their segregation and consequently discovering the cell-subpopulations.

Methods: In the current study, USCs were obtained from urine samples collected from six healthy donors and three DMD patients, having different deletions. Myogenic transformation, using the adenovirus type 5 (Ad5) was applied on USCs, derived from three healthy controls and one DMD patient, bearing the exon 45 deletion. Afterwards, in order to evaluate the gene expression profile in both native and myogenically transformed USCs, we performed RNA sequencing analysis using illumina Hiseq4000. Furthermore, USCs obtained from both healthy individuals and DMD patients, with recognized mutations, were subjected to the segregation, through the use of the Celector ® (StemSel Ltd); a new apparatus to distinguish cells, according to their physical features, such as shape, density, dimension etc. Following this, a flow cytometry was performed in order to test the mesenchymal stem cell markers. Then, the DMD transcript of the cells was profiled, using FluiDMD; a TaqMan gene expression assay.

Results: This study demonstrated that subsequently to induced myogenesis, many pathways correlated to DMD were modulated, examples of that are: muscle function and inflammation. Furthermore, a clear heterogeneity between, healthy and DMD USCs, regarding the cell-type constitution was spotted through the use of RNA seq. We also characterized USCs and consequently unveiled the presence of 3 three distinct sub-populations with particular physical and expression features using the new cell sorting

technique Celector®. Consequently, we assumed that USCs are extremely versatile and heterogeneous.

Conclusion: USCs are a very useful, in vitro cell model, to profile transcriptomics in DMD pathology and, thus, facilitate functional studies aiming at addressing drug efficacy.

ABSTRACT ITALIANO

Basi scientifiche: Al giorno d'oggi, le cellule staminali derivate dall'urina (USC) sono considerate una nuova base per modellare un'ampia varietà di malattie umane. In realtà, queste linee cellulari hanno molti vantaggi in termini di isolamento facile, bassi costi e assenza di problematiche etiche.

Scopo: Gli obiettivi di questo studio includevano l'individuazione del profilo di espressione genica delle USCs sia native che trasformate miogenicamente (MyoD) derivate da pazienti con DMD e individui sani . Inoltre, abbiamo approfittato della nuova tecnologia Celector® (StemSel Ltd.) per distinguere sia le USCs di controllo che quelle DMD attraverso la loro segregazione e quindi capire le sottopopolazioni cellulari.

Metodi: In questo studio, le USCs sono state ottenute da campioni di urina raccolti da sei donatori sani e tre pazienti con DMD, con delezioni differenti. La trasformazione miogenica utilizzando l'adenovirus di tipo 5 (Ad5) è stata applicata su USCs derivate da tre controlli sani e un paziente con DMD che aveva la delezione dell'esone 45. Successivamente, al fine di valutare il profilo di espressione genica nelle USCs sia native che miogenicamente trasformate, abbiamo eseguito l'analisi di sequenziamento del RNA utilizzando illumina Hiseq4000. Inoltre, le USCs ottenute sia da individui sani che da pazienti DMD con mutazioni riconosciute hanno subito la segregazione attraverso l'uso del Celector® (StemSel Ltd) che è attrezzato per distinguere le cellule in base alle loro caratteristiche fisiche quali forma, densità, dimensione ecc. Oltre questo, è stata eseguita una citometria a flusso per testare i marcatori delle cellule staminali mesenchimali e di conseguenza il trascritto DMD delle cellule è stato profilato utilizzando FluiDMD, un saggio di espressione genica TaqMan.

Risultati : Questo studio, ha dimostrato che successivamente alla miogenesi indotta, sono state modulate molte vie correlate al DMD, esempi di ciò sono: funzione muscolare e infiammazione. Inoltre, è stata individuata , attraverso l'uso di RNA seq, una chiara eterogeneità tra USCs sani e USCs DMD, per quanto riguarda la costituzione di tipo cellulare. Abbiamo anche caratterizzato le USCs e di conseguenza abbiamo svelato la presenza di 3 tre sottopopolazioni distinte con particolari caratteristiche fisiche ed

espressive utilizzando la nuova tecnica di smistamento cellulare Celector®. Di conseguenza, abbiamo ipotizzato che le USCs siano estremamente versatili ed eterogenei.

Conclusione: Le USCs sono molto interessante e utile per studiare il trascrittoma DMD e per facilitare la valutazione di efficacia di nuovi farmaci.

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I. Introduction and Literature Review

1.1. Stem cells

Stem cells are non-specialized cells, usually, characterized by remarkable properties, including their unlimited self-replication. In addition to that, under certain circumstances and using specific signals, they can differentiate into a vast number of mature cells [2].

Stem cells exist under different categories, such as embryonic stem cells (ESCs), adult stem cells and induced pluripotent stem cells (iPSCS). These cells, can generate different lineages: hepatocyte-like cells, neurons, brain, cardiomyocytes, muscle, hematopoietic cells and retina**.** Lately, stem cells have drawn much attention in both basic research and clinical therapy [3].

1.2. Classification of stem cells

1.2.1. Embryonic stem cells (ESCs)

The fertilization process, at the very early stages, leads to the formation of the so- called, "mass blastocyst" the internal wall of which, is bound to embryonic stem cells. Blastocysts are composed of two main parts: the inner cell entity or (embryoblast), that results in the formation of the fetus, and the trophectoderm that develops into trophoblasts (outer cell mass) and then, to placenta [2].

ESCs, hold great promises for innovative therapies for a various number of diseases, thanks to their great potential, manifesting in their illimited proliferation and pluripotency, enabling them to transform into almost any cell type [4]. Regardless of that, unethical issues, related to the manipulation of the early embryonic cells, in addition to their ability to trigger the immune response, remain the main concern for their usage by the scientific community [5].

1.2.2. Adult stem cells

At the end of the development, adult stem cells (somatic cells) are undifferentiated cells, that are found in a broad range of differentiated tissues in the body, such as skin, blood and neurons. Somatic cells take a longer period to expand then ESC.

They are considered of major importance, because they are self-renewable and are involved in many different roles, such as development, repair and restoration of cells that are lost on a daily basis, by injury etc. Consequently, homeostasis inside the human body is regulated by adult stem cells. A major disadvantage of this kind of cells, consists in their limited differentiation potential [2].

1.2.2.1. Mesenchymal stem cells (MSCs)

Definition and characteristics

Mesenchymal stem cells (MSCs), are a type of self- renewable somatic cells, characterized by their multipotent differentiation and proliferation capacity. As soon as they were discovered, in the 70ies, by Frienstein, it was assumed that they originate uniquely from bone marrow. In culture, MSCs, have been first described as easily handled fibroblast-like cells, with some interesting features, like growing into substantially forming clonogenic (colony-forming unit)-fibroblast (CFU-F). Furthermore, mesenchymal stem cells, were found to be adherent to plastic, with the potential to reach expansion in vitro over a remarkable number of passages. MSCs have the ability to differentiate into mesoderm-type cells like myocytes, chondrocytes, osteoblasts and adipocytes [6-8].

Currently, it is agreed that MSCs, can be actually harbored, inside a numerous variety of tissues, like: adipose tissue, peripheral blood, dental pulp, yellow ligament, menstrual blood, endometrium, milk from mothers, as well as fetal tissues such as amniotic fluid, membranes, chorionic villi, placenta, umbilical cord, Wharton jelly, and umbilical cord blood [9, 10].

In recent decades, the biomedical applications of mesenchymal stem cells (MSCs), have attracted increasing attention, due to the fact that they can differentiate into various mesodermal cells , such as osteoblasts, chondrocytes, muscle cells, and adipocytes [1] **(Fig.1.1)**.

Phenotypical characterization

In spite of the fact that, until now, there are some controversies, regarding the nomenclature of mesenchymal stem cells because they share many surface markers with other populations. Many research papers, and also clinical applications, consider the

International Society For Clinical Therapy (ICST) criteria ,set in 2006 for MSC, as the actual standard and seem to be dependent on these norms as being adequate to characterize these population that are still under examination [11].

According to, the ICST and the International Federation of Adipose Therapeutics and Sciences (IFATS) , MSCs have to express CD90, CD29, CD44, CD51, CD105 , CD166, CD49a-f, CD73 , CD106, and Stro-1 and lack of expression of CD45, CD34, CD14 and HLA-DR surface molecules [12].

These properties led to development of progressive methods for isolation and characterization of MSCs from various sources for therapeutic applications, in regenerative medicine [13].

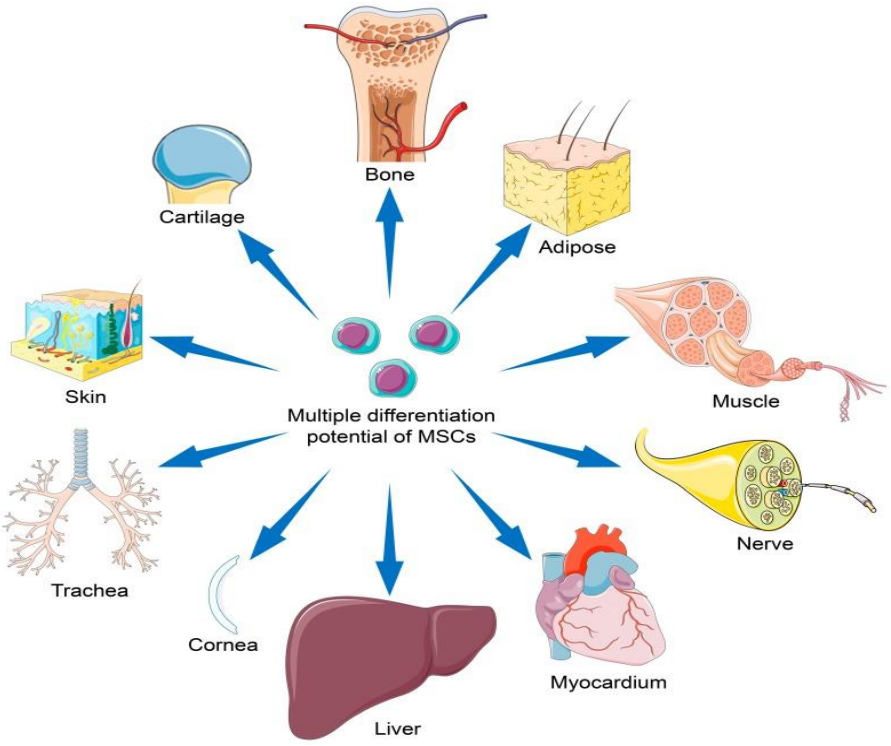

Figure 1.1: The different possible uses of Mesenchymal stem cells (MSCs) for the regeneration of a wide variety of organs [1].

1.2.2.2. Induced Pluripotent stem cells (iPSCs)

Over the course of the past decade, the most valuable achievement, leading to the significant advances in science, was the development of the induced pluripotent stem cells by Shinya Yamanaka in 2006 [14].

It was assumed that, iPSCs are characterized by self-renewal and pluripotency. Along with that, they can be obtained from multiple cell types, with different reprogramming potential, which in turn, depends on the specific cell line type. Consequently, iPSCs are considered as a great mean for a wide variety of research, thanks to their ability to differentiate into any cell type [9, 10, 15].

iPSCs are seen as an excellent therapeutic tool for cellular modelling, in neuromuscular and neurodegenerative diseases and, to help, treat many life-threatening illnesses, that are presently incurable. In fact, many emerging techniques were established aiming to reprogram somatic cells into iPSCs. Despite the fact that, the latter cells are regarded as an interesting future tool, some issues such as the invasiveness of the techniques essential to harvest the cells, their tumorigenic potential, the abiding manipulation , the insufficient reprogramming and poor differentiation , remain the main hurdles that interfered with their usage in the clinical field [15-17].

Therefore, urine derived stem cells (USCs) are regarded as a better alternative and promising source, thanks to their non-invasive, easy and low expense methods of isolation [18].

1.3. Urine derived-stem cells (USCs)

1.3.1. The origin of USCs

Most researchers, have hypothesized that, since USCs exhibit a strikingly, high gene expression, for cortex markers, localized on kidney, the latter represents the most probable origin niche for these cells. A study, made by Bhardwaj and colleagues; has shown that after a kidney transplantation, from a male donor to a woman, the USCs from the latter, enclosed the Y chromosome and displayed the usual kidney cell markers (PAX2 and PAX8). This, further confirms that USCs derive either from the kidney or the urinary tract. Additionally, USCs from voided urine has shown the exact same morphological,

phenotypic and growth features and the same differentiation capacity as the USCs obtained from the upper urinary tract, which in turn, proves that, these cells originate from the upper urinary tract. It has been also demonstrated that, USCs are recognized by specific genes, protein markers (synaptopodin and podocin) as well as renal markers, such as sine oculis home box homologue 2 (SIX2) or the neural cell adhesion molecule (NCAM) [19, 20] .

1.3.2. Characteristics

USCs, are usually defined as a recently discovered, subpopulation of cells that is, isolated from human urine samples, displaying many MSCs markers, such as CD73, CD44, CD105, CD90. On the other hand, they do lack the hematopoietic stem cells markers CD45, CD34, CD31 and HLA-DR [21, 22].

Despite the fact that, USCs markers, are similar to those of the MSCs , but the former ones, also express pluripotent stem cell markers, including POU5F1 or Oct 3/4, c-Myc, SSEA-1/4, and Klf-4 [23, 24] as well as pericyte (CD146), endothelial (vWF), epithelial (Ck 7,Ck13, Ck20 and AE1/AE3), smooth muscle (alpha-sma, Desmin) and interstitial (c kit) markers [22].

Besides, Bhardawaj and colleagues, have described through literature, that the telomerase activity, has reached 60% in different USCs samples, and they stated that the normal karyotype , was found until the 15th cell passage , indicating the absence of tumorigenicity [19].

Concerning the morphology, it was reported that USCs are not a single cell population and thus, are composed of different subpopulations. Two morphologically different cell subpopulations, were recognized and characterized. The first one, is spindle shaped, meanwhile the second has a rice-like morphology. Both subpopulations have shown multipotency and high expression levels of stemness-related genes [25].

1.3.3. Applications

Urine derived stem cells (USCs), possess an outstanding potential to differentiate into mesenchymal derivatives , such as osteoblasts, chondrocytes , adipocytes [26], along with their ability to be transformed to neurons, smooth muscle cells, as well as to, myogenic,

endothelial and urothelial cells under specific conditions and induction culture (**Figure 1.2)** [19, 27].

Thanks to all these characteristics, much research is shedding the light on the importance of the USCs as an in vitro model to study disease mechanisms, to identify new biomarkers, to test compounds and to use gene editing methodologies [28]. An important thing to be outlined, is that, besides the use of native USCs, some manuscripts have tackled the importance of the reprogramming techniques, that are based on obtaining iPSCs from patients - derived urine cells. Despite the fact that, their reprogramming efficiency can attain 4% which is still, relatively low, when compared to fibroblasts that were shown to be reprogrammed to a level that can reach up to 90%[29, 30]. Many advances, can still be made, in order to address the issues faced by these cells.

Recently, *Slats* et. al were the pioneers, to have established, the prospective use of urine-derived cells as a cellular model to diagnose and explore the underlying pathophysiological mechanisms of the patients, suffering from Fabry Disease (FD), an X-linked inherited disorder, which is the outcome of mutations, in the alpha-galactosidase (GLA) gene. Consequently, this affects the alpha-galactosidase A (α -GalA) enzyme, that becomes deficient and results in a buildup of globotriaosylceramide (Gb3). It was reported that, urine stem cells, derived from patients suffering from FD, display a low α-GalA enzyme function and Gb3 agglomeration, when, compared to control urine cells. Therefore, the forthcoming measurement of the enzymatic activity, in urine derived cells, can become an essential step for the future diagnosis and prognosis of the Fabry disease [31, 32].

In a similar trend, the isolation of USCs was also reported in the work of *Schosserer* and colleagues, for the treatment of the inherited epidermolysis bullosa (EB) ; a group of heterogeneous diseases caused by the mutation of not less than 17 genes that encode for intracellular; transmembrane or extracellular proteins [33].

Likewise, urine cell cultures were, also obtained from patients, suffering from spinal muscle atrophy (SMA), and were reprogrammed into urine induced pluripotent stem cells (UiPSCs), with the aim of substituting the invasive muscle and skin biopsy. It was reported, that SMA patient-derived urine cells carried the SMN mutation and thus, USCs

can be considered as a beneficial tool for molecular studies and for screening of potential drugs to treat SMA [34].

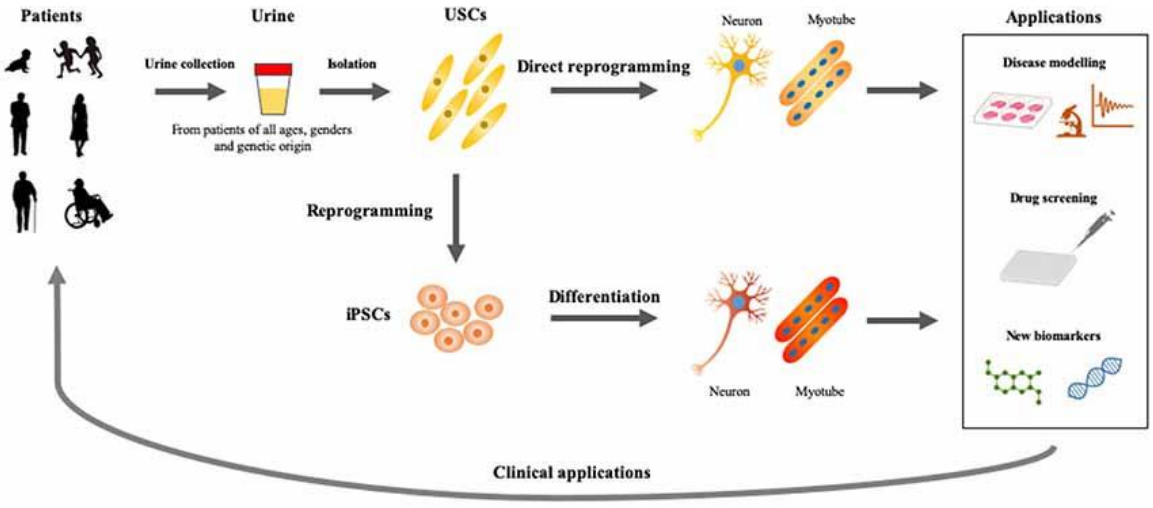

Figure 1.2: Diagram showing the easy isolation of USCs from all patients' samples regardless of their ages, gender and race. USCs constitute a great mean through their use as a cellular model for clinical applications either by direct reprogramming or by their transformation into iPSCs [35].

1.4. Duchenne Muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD), was first described in 1868 by Guillaume Benjamin Armand Duchenne [36]. DMD, is regarded as a lethal and one of the most common forms of the inherited muscular dystrophies, with an incidence of 1/5000 in male live births worldwide [37]. It is a disorder caused by inherited or spontaneous mutations of the dystrophin gene located , in the X chromosome, that results either in the absence or insufficiency in the functional dystrophin [38, 39]. The loss of dystrophin is responsible for; dramatic muscle deterioration, persistent damage in myofibers, chronic inflammation, progressive fibrosis and dysfunction of muscle stem cells. Many efforts, have been made in the last decade in order to improve the diagnostics and therapeutic status for DMD but current treatments do not completely cure the disease [40].

1.4.1. Dystrophin gene and protein

The dystrophin gene or DMD is the largest human gene, identified, so far and is positioned, on the short arm of the X chromosome in the region Xp21, containing 79 exons [41].

The DMD gene is characterized by a sophisticated organization of 7 different promoters that lead to the transcription of the full-length isoforms: Dp427c (cerebral), Dp427 m (muscle) and Dp 427p (purkinje) dystrophin. Furthermore, four different shorter isoforms Dp260 retinal (R), Dp140 brain-3 (B3), Dp116 Schwann cell (S) and Dp71 general (G) are also generated from 4 different internal promoters [42].

Dystrophin, is a large 427-kDa rod-shaped cytoskeletal protein [43]. It is distinguished by having, four major domains: an NH2-terminal actin binding domain, the central domain, the cysteine-rich domain, and the COOH-terminal domain (**Figure 1.3)** [44-46].

Dystrophin, is usually defined as a cytoplasmic protein that is connected to inner surface of the sarcolemma, which attaches to different cytoplasmic and transmembrane proteins of the dystrophin-glycoprotein complex (DGC), and plays a major role as a cytoskeletal integrator inside the complex [47, 48].

Based on this structure, it is generally assumed, that the main function of dystrophin, is the transmission of force, laterally, across the muscle and/or helping to maintain the connection between the membrane and intracellular cytoskeleton matrix and extracellular matrix [49].

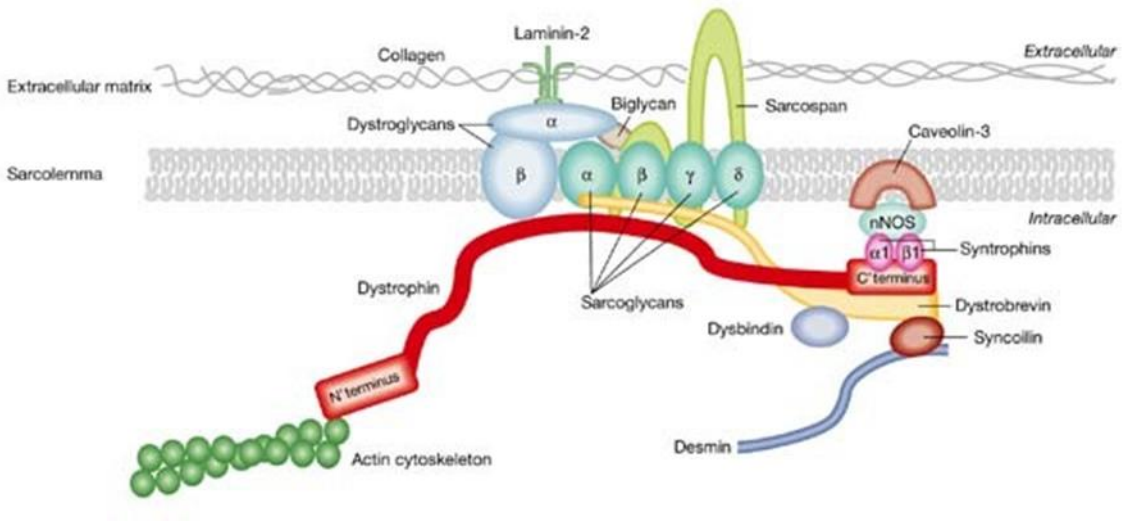

Figure 1.3: Scheme displaying the structure of the dystrophin protein complex that connects the internal cytoskeleton to extracellular matrix [50].

1.4.2. DMD mutations and severity of the disease

Mutations in the dystrophin or the so–called, DMD gene are responsible for the dystrophinopathies Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), and X-linked Dilated Cardiomyopathy (XLDC). Throughout history, due to the extremely large size of the gene, the detection of the DMD mutations has been very challenging [51].

The most predominant cases that constitute almost 75% are called large rearrangements, and manifest as deletions and duplications. Conversely, the other mutations are either small mutations (nonsense or frame-shifting mutations) 25%, or sporadic deep intronic copy number variants (CNVs)/small mutations [52, 53].

It has been reported, that usually, the relationship between the genotype and the phenotype matches up with the reading frame rule or the Monaco rule. A further explanation of this, is that, the severe DMD phenotype, can be the result of the mutations, that affect the translational open frame, and that, consequently cause the dystrophin protein deficiency. On the contrary, the BMD phenotype, corresponds to a shorter protein that results from the type of mutations, in which the reading frame is not altered [54]. Nevertheless, there are always some exceptions to the rules, as described by Le Rumeur. Surprisingly, an in-

frame mutation , might also be the reason , behind a severe DMD pathology [55]. Despite the fact that the whole DMD gene, is susceptible to alterations, hotspot mutations are commonly detected. As previously reported, duplications are perceived from exons 2 to 10 while deletions, usually occur between exons 45 to 55 [53, 56].

As previously stated by Ferlini and colleagues, sporadic DMD complex rearrangements may also occur. The latter mutations, often lead to a new, cryptic site which brings a new (cryptic) splice site inducing exon orientation inversion, and consequently, resulting in exon skipping [57].

Up to that time, it has been demonstrated that, a duplication is often the outcome of a grand paternal germline. Conversely, a high number of DMD patients with deletions, have mothers who are also carriers of the same mutation. Thus, a familiar trend is usually dominating in these kinds of mutations [58].

It is also important to outline the fact that, the geography and the race, have a great impact on the frequencies of the mutations. Indeed, epidemiological studies were carried out in China, Spain, Italy and revealed the particular spread of DMD, specific mutations according to the evaluated region [40].

1.4.3. Therapeutic strategies

So far, many approaches have been examined, in order to treat DMD and can be divided into two groups: therapies that aim to slow down the symptoms of the disease such as the steroids, and those that target the repair of the dystrophin expression and consequently intend to cure the DMD. The latter type includes : utrophin modulation, stop -codon read through therapy, vector-mediated gene therapy, cell therapy and exon skipping[59].

The steroids, are considered one of the most historically known first line treatments. Prednisone and deflazacort are the commonly used corticosteroids, and were shown to be very efficient for DMD patients, exerting a positive effect on the muscles, in terms of strength and function [59-61].

Another therapeutic strategy, for the treatment of DMD patients, is known as, utrophin modulation. The new drug called Ezutromid (SMT C1100), recognized as a utrophin modulator is still currently under phase II of the clinical trial [62].

On the other hand, in order to reestablish the dystrophin expression, many different approaches are used such as the stop- codon read through therapy. In fact, for this kind of treatment, PTC124 (Ataluren, TranslarnaTM) has shown its effectiveness and safety in a Phase II trial and is currently , conditionally approved in Europe. [63] . Lately, many improvements have been achieved, regarding the gene therapy which is based on the use of the adeno-associated virus (AAV)-mediated mini-/micro dystrophin transfer. Actually, in 2020 a non-randomized control trial was conducted, in order to assess the systemic delivery of rAAVrh74.MHCK7 micro-dystrophin. Although results were promising, in terms of safety and appropriate delivery, the outcomes of this study, are to be acknowledged in a randomized clinical trial[64].

Additionally, cell therapy approaches, have also been tested using a wide variety of cells, in the purpose of regenerating the patient's damaged tissue or organ through either a heterologous or autologous cells transplantation. Further advancements and progresses are still being made to overcome the hurdles of this technique [65].

One of the most successful therapy for DMD is exon skipping. The concept of this strategy lies in skipping particular exons, in certain DMD mutations, through the administration of 20-30 bp of synthetic antisense oligonucleotides (AON) which lead to the modulation of the dystrophin pre-mRNA, splicing process. Hence, AON bind, specifically to splice motifs essential for pre-mRNA processing and consequently lead to masking of the splicing signals on the RNA. Thus, both introns and the adjacent exons are excluded [66] (**Fig 1.4**).

Therefore, the severe DMD is transformed into the milder BMD phenotype ,after the formation of a functional protein ,although it is shorter in size [66]. The main advantage of exon skipping, is that almost 80% of patients ,with deletions can undergo this kind of treatment [67]. It has been known, that the DMD mutation hotspot is clustered between exons 45 to 55, as well as, between exons 2 and 10. Due to the fact that, exon 51 skipping

is actually applicable on 14% of DMD patients, the development of a drug that targets skipping of the exon 51, was of major importance [41].

The 2 major successful, antisense oligonucleotides, used for exon 51 skipping, in clinical trials are: the 2'-O-methyl-phosphorothioate oligonucleotides (2'OMePS) also known as Drisapersen (developed by Prosensa), and the phosphorodiamidate morpholino oligomer (PMO) named Eteplirsen (developed by Sarepta Therapeutics). Both of them, are characterized by their high stability, high efficacy, and low toxicity. It is notable that, the two drugs are chemically diverse [68]. Nowadays, the exon skipping strategies are much wider and more exons are targeted by PMO; an example of that is the drug targeting exon 53 that is still, under investigation, although it was shown to be completely safe and able to induce exon 53 skipped mRNA in all patients [69]. In addition to that, clinical trials to skip exon 45 by PMO are currently ongoing https://www.sarepta.com/pipeline/exon-skipping-duchenne. The outcomes of exon skipping, are very encouraging and more studies need to be held in order to expand the number of drugs, that could target the biggest number of exons possible.

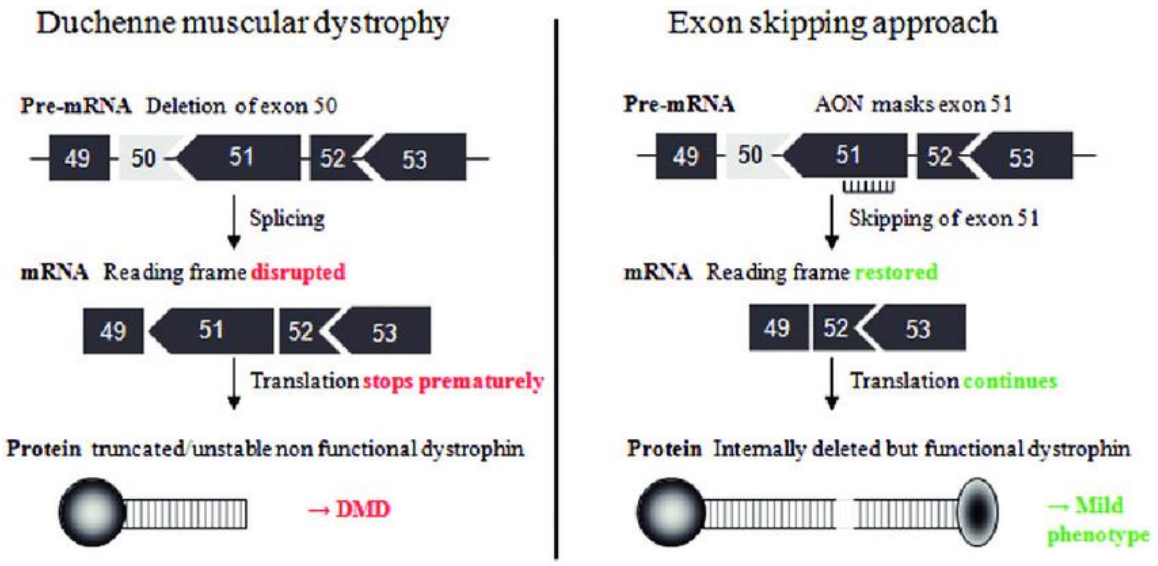

Figure 1.4 : Scheme showing the principle of the antisense exon-skipping strategy for DMD. In the case above, the patient has exon 50 deletion which has led to the alteration of the reading frame and thus the generated dystrophin protein is unstable and non-functional (Left Panel). The treatment with AON for exon 51 skipping results in the restoration of the reading frame , and

consequently although the obtained dystrophin protein is internally deleted but it maintains part of its function (Right Panel) [70].
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1.5. Urine-derived stem cells and Duchenne muscular dystrophy

Neuromuscular and neurodegenerative diseases are commonly modeled, using genetically modified animals such as mice. Yet, animal models do not recapitulate all the phenotypes that are specific to human disease.

Currently, scientists have shed the light on a new approach that consists of the cellular modelling of human disease. This strategy is very beneficial because it is able to recapitulate the disease phenotype in vitro, which helps understanding the pathophysiology of the different neuromuscular and neurodegenerative diseases. This, enables us to reach a personalized medicine tailored for each patient. Nonetheless, up to that date, choosing the perfect type of cells ,is still a topic of debate [35].

Concerning the Duchenne muscular dystrophy, primary myoblasts were perceived, as a convenient tool, to model the disease, since they express a high level of mRNA. However, the main disadvantage about this cell source , lies in the invasive procedures needed to get a muscle biopsy from patients [35].

Fibroblasts, are considered another cellular model, that can be employed in the study of DMD, through their transformation into myotubes, by MyoD1 transduction. Yet, the skin biopsy to obtain these cells, is invasive, especially in children. Furthermore, the amount of dystrophin mRNA and protein is usually not enough [71]. The previous challenges, were defeated by the new approach, known as Human-induced pluripotent stem cells (HiPSCs). The latter cellular model constitutes an important supply for cell types considered rare or inaccessible ,such as skeletal and cardiac myocytes [35].

In spite of this, many restrictions are encountered with this technique, example of that are the biological diversity between iPSC lines and differentiated cells, the genome instability and the epigenetic memory ,associated with reprogramming iPSCs, in addition to modeling of diseases ,that are, epigenetically influenced by environmental factors [35].

Therefore, taking into account that, cells used in vitro should be easily accessible from all patients regardless of their gender, race, or even age, using a simple noninvasive and low-cost procedure, seems to be of great importance. Accordingly, researchers, have recently elucidated the importance of urine, which, is an effortless available source, enclosing cells with stem-like characteristics and since then, they were recognized as a useful tool in disease modelling [28, 72].

Urine -derived stem cells, are considered nowadays a powerful modeling tool for human specific diseases and for generating pluripotent stem cells lines. Despite this, very few studies, have investigated the importance of urine- derived stem cells, in drug screening and disease modeling. In a report, Zhou and colleagues, have generated iPSCs from urine-derived stem cells. It was consequently demonstrated that, iPSCs, generated from urine -derived stem cells (UiPSCs), are better than iPSCs, obtained from somatic cells, since they can be induced in a short amount of time [15, 73].

Furthermore, some studies concerning skeletal muscle diseases , have tackled the direct reprogramming of USCs into myogenic cells, demonstrating that the USCs derived from patients with DMD, can maintain the specific mutation [22, 74]. Also, recently, a novel MyoD1-converted, urine- derived cell model was developed in vitro to model the pathological processes of muscle cells affected by DMD [75].

This in turn, demonstrates that USCs, can ,be actually employed in a variety of research purposes to get a deeper view of the of the pathophysiological aspects of several diseases which ,consequently leads to the identification of new therapeutic approaches, for patients [35].

Aims of the study

The emanating, urine-derived stem cells (USCs) are considered as a new source to better diagnose and model human diseases [28]. Yet, many characteristics of the former cells, are still unclear.

In this study, we intended to deeply characterize DMD, and control USCs and depict:

1. The transcript profile of both native and myogenically (MyoD), transformed USCs from healthy subjects and DMD patients, through the use of RNA sequencing.

This, may outline the importance of the USCs, in the interpretation of the DMD gene mutations and revealing the causative mechanisms.

2. The heterogenous cell composition of USCs, using the new tool Celector ® (StemSel Ltd.) which is efficient to identify, sort and profile, the different sub-populations.

This, allows us to have a deeper insight on the different characteristics and the possible applications of this cellular model. Additionally, Celector® might be implicated in the separation of particular cells displaying diverse features, bringing about a wide variety of cell lineages, the fact which can become a milestone in both therapy and research.

The major purpose of this research, is to confirm the viability of the USCs for transcriptomic profiling. In our case, we tested the myogenically transformed DMD USCs in order to test whether they represent the perfect model to study the DMD disease.

In this context, we can say that USCs could represent the perfect candidate for profiling the RNA and for the interpretation of the DMD disease patterns.

II. Material and Methods

2.1. Entities registered in the study

In the **Table 2.1** we presented the appropriate details for each person, enrolled in the study. Informed consent was obtained from all the individuals (controls and DMD patients) (UNIFE Ethical Committee approval, N. 161299 (20/05/2020) and N. 66/2020 (23/01/2020).

USCs from healthy donors (Control 1-6) were isolated as control cell lines. USCs from DMD patients, with known mutation and confirmed genetic diagnosis (DMD samples IG, IH, and AF carrying the deletion of exons 45, 50-52 and 46-47, respectively) were used, for RNA sequencing or Celector® analysis **(Table 2.1)**.

Sample	Disease	Mutation	Gender	Urine processing	USCs application
DMD IG	DMD	del ex 45	M	within 4 hours	RNA sequencing
DMD IH	DMD	del ex 50-52	M	within 4 hours	Celector separation
DMD AF	DMD	del ex-46-47	M	within 4 hours	Celector separation
C-1	Healthy Control	/	M	within 4 hours	RNA sequencing
C-2	Healthy control	/	M	within 4 hours	RNA sequencing
C-3	Healthy Control	/	M	within 4 hours	RNA sequencing
C-4	Healthy control	/	M	within 4 hours	Celector separation
C-5	Healthy control	/	M	within 4 hours	Celector separation
C-6	Healthy control	/	M	within 4 hours	Celector separation

Table 2. 1: Representation of the different DMD patients and healthy controls registered in the study to obtain samples of urine required for USCs isolation.

2.2. Human USCs Isolation

The **Table 2.1** represents all the individuals from which urine samples, were obtained. As described by Falzarano and colleagues [22], USCs were derived and cultured accordingly. In brief, a first morning urine and a second urine sample were collected from each subject and processed within 4 hours from the collection.

2.3. Myogenic transformation using MyoD

As previously described, through the infection with adenovirus serotype 5 (Ad5)-derived, EA1-deleted adenoviral vector carrying the MyoD gene [76], we induced myogenesis on USCs obtained from DMD patient IG and control individuals. MyoD transformed USCs were differentiated, into myotubes, by serum deprivation.

2.4. The analysis of the dystrophin gene expression of USCs using RNA-seq

We analyzed the gene expression levels of i) native USCs, derived from a pool of 3 healthy donors (C-*n*) and from the DMD patient IG (IG-*n*), ii) MyoD-transformed USCs from controls (C-*m*) and from the DMD patient IG (IG-*m*) **(Table 2.2)**.

Total RNA was isolated from native and MyoD-transformed USCs of DMD patient IG and healthy controls using the RNeasy-kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Libraries were prepared, using TruSeq Kit (Illumina) according to manufacturer's instructions.

Quality and quantity of the RNA library, was assessed using, the Agilent RNA 6000 nano kit on an Agilent 2100 Bioanalyzer and the ABI StepOnePlus Real-Time-PCR System. The RIN (RNA Integrity Number) for all analysed samples was from 9 to 10 **(Supplementary Materials)**.

RNA sequencing was carried out with Illumina HiSeq4000 at the Beijing Genomics Institute (BGI, Beijing). Read quality was accessed using fastQC (v. 0.11.3; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were trimmed for adapters and for length at 100 bp with Trimmomatic, resulting in about 22M (range 14.8M-31.7M) trimmed reads per sample **(Table 2.3)**. Reads were subsequently aligned to the human reference genome (GRCh38) using STAR (v. 2.5.3a; [77]). Raw gene counts were obtained in R-3.4.4 using the *featureCounts* function of the *Rsubread* R package (v. 1.30.3; [78]) and the Gencode gene annotation. Raw counts were normalized to counts per million mapped reads (CPM) using the *edgeR* package [79]; only genes with a CPM greater than 1 in at least 1 sample were further retained for differential analysis, for a total of 20,716 genes. Differential gene expression analysis was performed using the *exactTest* function of the *edgeR* package [79]. Genes were considered significantly differentially expressed at FDR≤0.050. Functional enrichment analysis was performed using Gene Set

Enrichment Analysis (GSEA, v.4.0.3; http://software.broadinstitute.org/gsea/index.jsp) software and gene sets derived from Hallmark, Reactome, Kegg and Gene Ontology collections of the Molecular Signature Database (MsigDB, v. 7.1; https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). The GSEA algorithm was applied using the signal2noise metric and the weighted statistics: gene sets were considered significantly enriched at FDR ≤0.05 when using 1,000 permutations of the gene sets.

Sample abbreviation	Sample comparison
C-*n* RNA pool from *native* USCs of control 1-3	C-*m* vs C-n
IG-*n* RNA from *native* USCs of DMD IG	IG-*n* vs C-*n*
C-*m* RNA pool from *MyoD*-induced USCs of control 1-3	IG-*m* vs C-*m*
IG-*m* RNA from *MyoD*-induced USCs of DMD IG	IG-*m* vs IG-*n*

Table 2.2: The analysis layout of RNA-seq and the comparison between different samples of USCS.
Each abbreviation represents a specific sample as used in the text (first column) and displays the comparison between the transcript profile assessed for each pair (second column).

Sample ID	raw fastq	trimmed	aligned: uniquely mapped	counted: assigned to coding regions
C-m	20,600,102	20,065,900	18,135,177	15,567,734
C-n	59,505,396	31,751,714	30,011,295	26,706,107
IG-n	32,956,669	22,756,422	21,872,447	19,532,133
IG-m	31,431,592	19,914,094	16,951,806	14,547,339

Table 2.3: Representation of the different Reads 'numbers obtained from the analysis through RNA-seq in all samples subjected to analysis.

2.5. Celector® technology for the Identification and separation of USCs

A label-free technology named Celector® (Stem Sel s.r.l., Italy) (**Figure 2.1**) was used in order to sort and analyze USCs cultures at low passage (i.e., p1-p2) [80].

Despite the fact that, in few cases, the cells aggregation can lead to some limitations in the analysis process, such as in the Flow Cytometry technique. This novel technology is able to overcome these hurdles, by the separation of single cells, from aggregates having different sizes. Owing to the fact that, Celector ® is capable of preserving viable cells, in addition to the shape of both single cells and agglomerates, consequently, they can remain suitable, for a series of different studies. Celector ® was also shown to be efficient in the separation of mesenchymal cells from epithelial cells, without requiring enzymatic digestion or usage of antibodies.

The cells were isolated from DMD patients IH and AF and from healthy controls 4-6 (**Table 2.1**). The separation is based on the Non-Equilibrium, Earth Gravity Assisted Fractionation (NEEGA-DF) principles. Therefore, cells are pulled apart and eluted, according only, to their physical characteristics, such as morphology, dimension, membrane rigidity and density. The latter properties, have a great influence on the cell position, across the capillary device, which is, already generated by the two opposite forces: the gravity and lift forces (**Figure 2.2**).

Cells having different positions, possess different velocities and therefore, elute at different time, allowing the separation and collection of several sub-populations (**Figure 2.3, Figure 2.4)**. A camera with a microscopic object, placed at the outlet of the capillary channel and connected to an imaging software, records live images of eluting cells and plots the number of counted cells related to elution time in a fractogram (profile). Dimension inclusion/exclusion criteria are set by the operator, to refine the counting procedure. A decontamination procedure of the fractionation system by flushing cleaning solution, followed by a wash with sterile, demineralized water, is performed every day before starting. Subsequently, to block unspecific interaction sites on the plastic walls, a sterile coating solution is flushed at 1 ml/min. The system is then ready to be used, after filling it with sterile mobile phase. All solutions are provided by Stem Sel s.r.l. (Italy).

For each analysis, cells, were trypsinized, centrifuged at 1200 rpm for 5 minutes, and resuspended in appropriate volume of PBS, to obtain a concentration of 300,000 cells per 100 µl. This volume, was introduced into the system and analyzed at a flow rate of 1 ml/min. Based on the cell profile, samples were divided in subfractions. Cells from each patient sample, were run, several times to collect the higher number of cells for downstream experiments.

For every analyzed sample, the cell area of eluting cells, was quantified using the ImageJ software (https://imagej.nih.gov/ij/index.html). One representative captured picture for each fraction was used and the particle analysis plugin was applied.

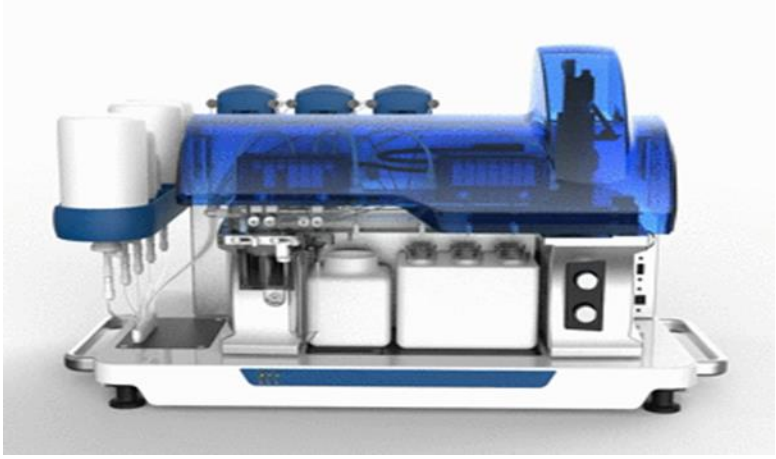

Figure 2.1: Celector® label-free technology. The principle is based on the separation of cells only on their native physical properties: dimensions, morphology and density (www.stemsel.it)

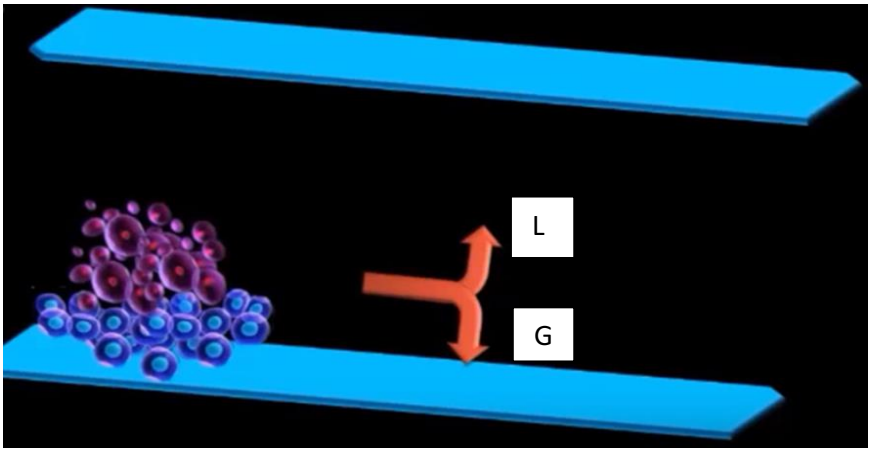

Figure 2.2: The combination of the gravitational (G) field and lift forces (L) for the different sub-populations of cells at defined height channel (www.stemsel.com).

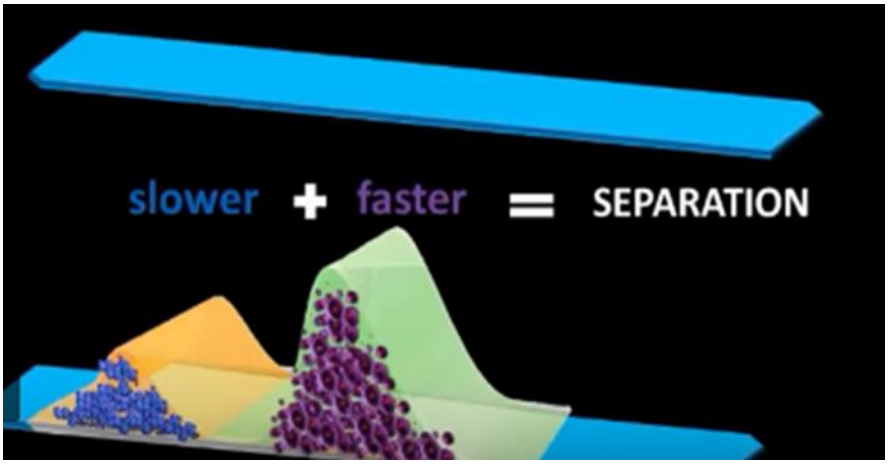

Figure 2.3: Cells are swept through the capillaries at different velocities (www.stemsel.it).

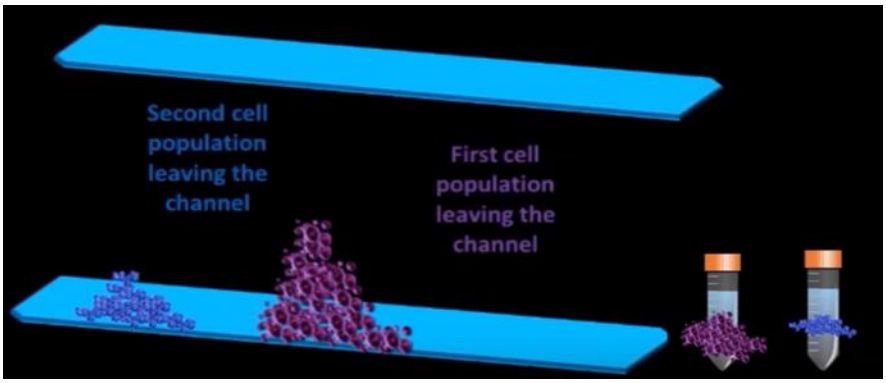

Figure 2.4: Different cellular populations are identified and collected as different fractions (www.stemsel.it)

2.6. USCs fractions phenotypic characterization

In order to proceed with flow cytometry and RNA analysis, cells for each fraction were collected and plated in expansion medium, for a further passage, to obtain the necessary cell number. The day after selection, cell images were acquired using a light microscope (Leica) to visualize morphological differences among fractions. As soon as cells reach confluency, they were trypsinized. 100,000 cells were stained for mesenchymal (CD90-Fitc, CD105-Pe, CD73-Fitc, CD146-Fitc) and hematopoietic markers (CD34-Fitc and CD45-Pe) and read using the FACS Canto (BD Biosciences). Data have been analyzed, using FlowJo software (FlowJo, LCC) and plotted in GraphPad Prism.

Total RNA was extracted from DMD USCs fractions, using the RNeasy-kit and reverse, transcribed into cDNA using random primers and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was performed on β-actin to verify cDNA synthesis.

The DMD transcript analysis was performed by FluiDMD cards [22] ,that profiles all exon-exon junctions of the 79 exons of the DMD transcript, including the DMD isoform promoter and/or first exon unique regions (B, M, P, Dp260, Dp140, Dp116, Dp71). FluiDMD cards were run on an Applied Biosystems real-time 7900HT appliance (Thermo Fisher Scientific). The cycle threshold (Ct) values, obtained for all exon junctions and

dystrophin isoform systems, were normalized, using human b-actin as the housekeeping gene (ΔCt = Ct exon junction system – Ct b-actin).

2.7. Statistical analysis

Statistical analysis was performed using the unpaired t-test. All data are shown as the mean with standard deviation (SD). Differences were considered significant when p-value is less than 0.05 (*p ≤ 0.05, ****p ≤0.0001). Genes were considered significantly differentially expressed at FDR≤0.050. ///FDR: p-value after false discovery rate correction. Only significantly (FDR ≤ 0.05) enriched gene sets are shown.

Unpaired t-test statistical analyses and plots of results, were obtained using GraphPad Prism software.

III. Results

3.1. RNA-seq approach marks out the DMD disease pathways in native and myogenic USCs

In order to evaluate the gene expression differences, between native and myogenically transformed cells and also between controls and DMD samples, we made a DEG analysis using the following: a) native USCs, derived from a pool of 3 healthy donors (C-n) and from the DMD patient IG (IG-n), b) MyoD-transformed USCs from controls (C-m) and from the DMD patient IG (IG-m). (**Table 2.2**).

Following myogenesis, a remarkable number of genes, has shown deregulation in MyoD-USCs, derived from controls (C-m) and DMD IG (IG-m) (2482 and 4583, respectively).
The analysis of the control samples revealed that, 909 genes were downregulated in C-m whereas 1573 genes were up-regulated in C-n (**Figure 3.1A**).
The same trend applies to the DMD sample; indeed, 1428 genes were supressed in IG-m, while 3155 genes were overexpressed in IG-n (**Figure 3.1A**). Our results, are consistent with the findings of a previous report [81]. In fact, it has been reported that in the course of the myogenesis induced by MyoD, numerous cellular activities, such as structural/cytoskeletal, cell cycle, metabolism and cell adhesion, are actually encoded by different genes that undergo deregulation during the former process.

It is noteworthy to mention that, the induction of myogenesis in both controls and DMD IG (C-m and IG-m) has mutually led to the intensification and up-regulation of gene groups, correlated to the muscular growth and basic function such as contraction, as shown in **Figure 3.1B**.

Comparing the natives DMD (IG-n) and control sample (C-n) we found a variability in genes expression. This is clearly shown in **Figure 3.1A** where 892 genes were subjected, to repression, in the former group versus an overexpression of 388, in the latter group. This deregulation becomes even more prominent, after the induction of myogenesis in both samples. In fact, IG-m displayed a down-regulation of 1155 genes, on the other hand C-m has shown an up-regulation of 1273 genes (**Figure 3.1A**). This stresses out the fact that, the particular transcriptional profile of the DMD USCs, is preserved and even more

emphasized after the myogenesis. This finding, is further confirmed by the Gene Set Enrichment Analysis (GSEA). In fact, the comparison of IG-m to C-m cells, unveiled an over-expression of most of the signals, that are implicated, with the muscle development, functional processes and differentiation, following the induction by myogenesis **(Figure 3.1B)**. In a similar trend, this was also reported to occur in DMD skeletal muscle [82].

Aiming to discover additional disease markers in USCs, by means of RNA-seq data, we also performed an in-silico deconvolution analysis. Consequently, inconsistency of gene sets enrichment related to cell type composition was detected among IG-n and WT-n.

A remarkable build-up of expressed genes for keratinocytes epithelial, mesangial cells and sebocytes was notable in control USCs. Interestingly, these markers indicate the most probable cell origin of USCs (i.e., glomerular parietal epithelial cells) [72]. Conversely, in DMD USCs, there was an enrichment of genes, coding for smooth muscle cells.
Thus, we employed cell sorting, to explore in-depth, the cell composition (**Figure 3.1C**).

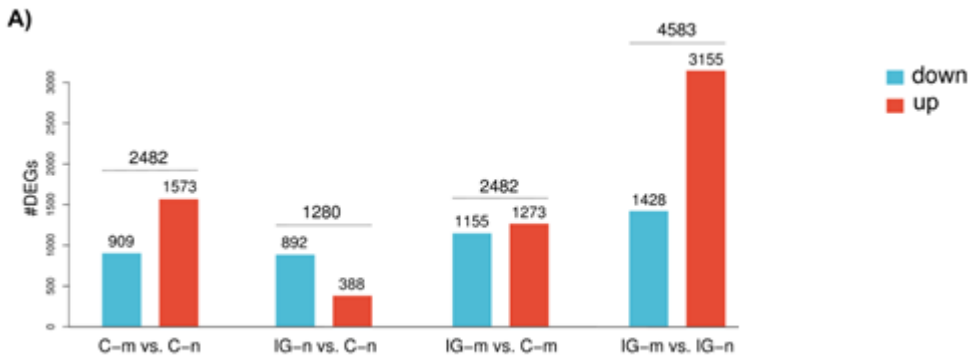

Figure 3.1.A: The differential expression of the number of genes in the variable tested samples; in myogenically transformed (m) and native USCs obtained either from controls (C) or DMD (IG).

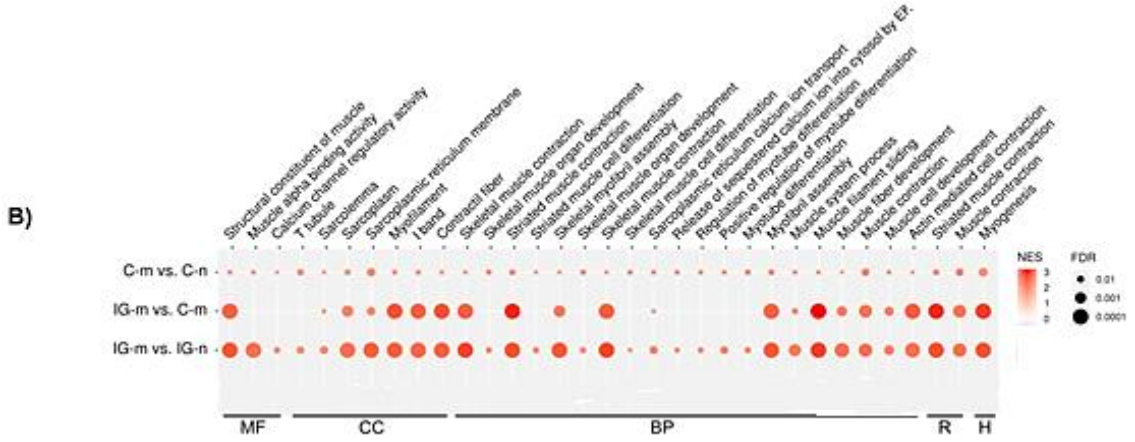

Figure 3.1.B: The myogenic transformation, in DMD and native USCs, induces the expression of the MYOD1 gene which triggers the transcription of several pathways associated to muscular activity/contraction, differentiation and development. MF: Gene ontology, molecular function; CC: Gene ontology, cellular component; BP Gene ontology, biological processes; R: Reactome gene set; H; hallmarks gene set. NES: normalized enrichment score. Red, positive NES, i.e., up-regulation, of the gene set in the first condition; FDR: p-value after false discovery rate correction. Only significantly (FDR ≤ 0.05) enriched gene sets are shown.

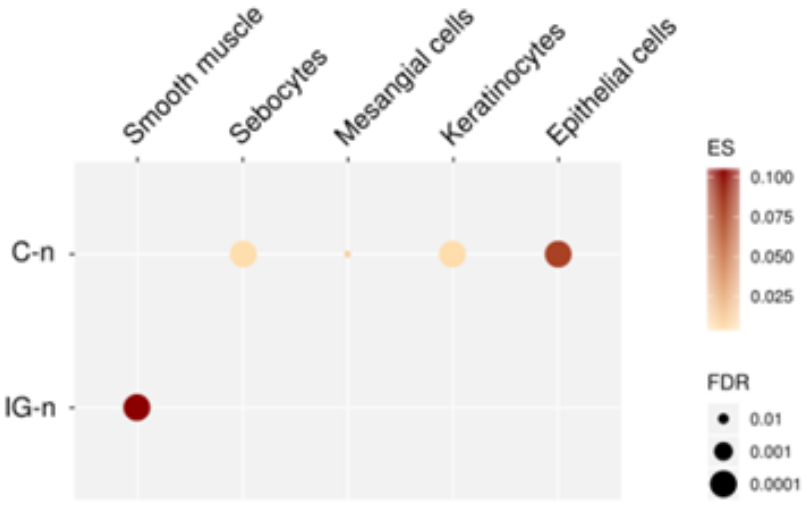

Figure 3.1.C: USCs from control samples show gene enrichment related to different cell types. Whereas, DMD cells are found to be homogeneous and enriched only in genes related to smooth muscle cells. ES; enrichment score; FDR: false discovery rate.

3.2. USCs Celector® analysis detects and segregates USCs sub-populations

The new technology Celector® was employed, aiming to categorize the USCs, obtained from the 3 different controls (C4, C5, and C6) and from the DMD patients AF and IH. Remarkably, samples profiling revealed heterogeneity between control and DMD USCs and also between the two DMD USCs.

Duplicability in the profile is what we recognized in the three control USCs (C4, C5 and C6). The results, showed the presence of two different peaks; a small one that extends from the 2nd to the 6th minute, followed by a big one (major peak), from the 6th to the 15th minute **(Figure 3.2A)**. The latter peak seemed to be made of two subfractions that are F2 and F3 **(Figure 3.2A)**, on the other hand, the small peak is uniquely distinct by the very first fraction F1 and is perceived as a population of heterogeneous, agglomerated cells **(Figure 3.2.D)**.

Alternatively, the examination of the DMD USCs, unveiled the presence of two different profiles. The analysis of the DMD IH, carrying the deletion of the exon 50-52 was

distinguished by the fact that, the elution of the majority of the cells was achieved before the 6th minute (colored area with an asterisk, **Figure 3.2B**). Whereas, the other DMD AF with the deletion of exon 46-47, displayed a major elution of cells after the 7th minute **(Figure 3.2B)**. It is notable that, the profile of the control samples, is generally diverse from the ones exhibited by the two patients which is clearly shown in **Figure 3.2 C**.

The size and density play a major role in the time of the elution. In fact, according to the NEEGA-DF principles, denser and bigger cells elute earlier than smaller ones. We noticed that the average of the area of the eluting cells in F2, for DMD patients is bigger than the one observed in the control cells, the same trend was detected concerning the fraction F3 **(Figure 3.2E)**.

A versatility was also noticed in the cell area, among fractions between the two patients. Surprisingly, USCs from DMD AF, both in F2 and F3 were bigger than those observed from the DMD IH, despite the fact that the latter has a wider cells distribution in the first section of the profile analysis **(Figure 3.2F)**. After the analysis, an assessment of morphology and phenotype examination, was achieved after cell collection from each sample followed by cell plating in a specific medium for their proliferation.

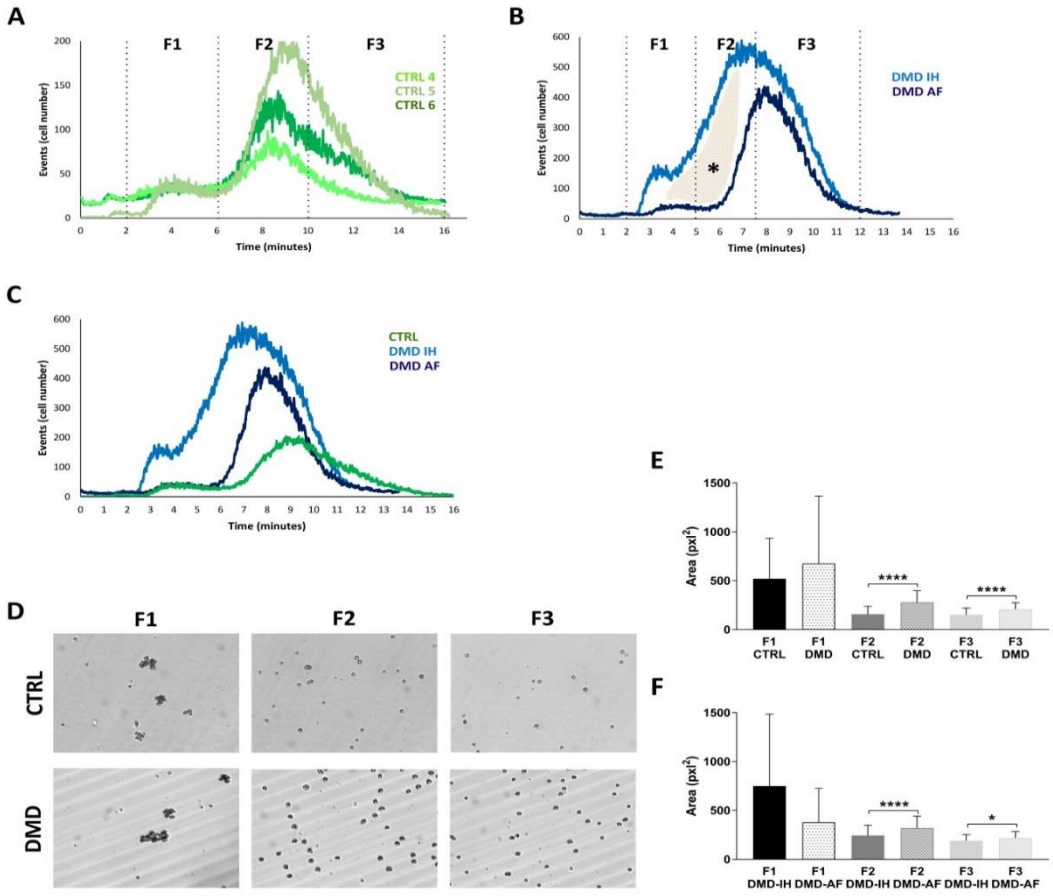

Figure 3.2 : Profile illustration of healthy donors (controls) (4-6) and DMD USCs (DMD 5, DMD 6) through the use of Celector®.

The first three panels (A, B, C), we can see the overview of separation and the fractions obtained from each sample (F1, F2, and F3). The number of eluted cells is illustrated versus time. **A)** The separation profiles of control cells are analogous; this can be detected from the identical time they take to elute from 6 to 15 min. The specific cell number acquired through the analysis is determined by the intensity of the signal and the peak height. Regarding the control pool, the very first peak represents the agglomeration of cells that were eluted at the beginning of the analysis (first minutes). **B)** When it comes to DMD cells, it is also remarkable that the separation profile is reproducible, although there are some differences in the time of elution (shorter) when compared to control cells. The comparison between the analysis of the two DMD samples has revealed that IH displayed an augmented dispersal of cells in the second fraction F2 (5 to 7,5 minutes) versus AF (colored area, *). **C)** A clear variability in the profiles of the Control and the two DMD samples is detected. The exit time of USCs from DMD samples from the capillary samples is quicker relatively to control. This suggests that the former cells are larger and have a higher density. **D)** Cell agglomerations are identified in F1 while single cells are found in F2 and F3, as shown by the images obtained by the camera located at the end of the capillary channel. **E)** Representation of the area of eluted cells. The DMD-USCs have a more increased cell area regardless of the cell fraction

(F1, F2 and F3), versus Control-USCs. **F)** Further variability in the area of the cells was also detected between both DMD. DMD IH exhibited smaller cells when compared to DMD AF (graphs showed average and standard deviation and unpaired t-test was performed: * $p< 0.05$, ****$p<0.0001$).

The morphology evaluation unveiled homogeneity, between the obtained fractions from all samples. The common characteristics of all analyzed fractions, consists in the ubiquity of fibroblast-like or rounded shapes.

DMD USCs displayed larger size than control USCs **(Figure 3.3A)**. Many manuscripts have demonstrated the presence of mesenchymal markers on USCs [21, 22, 29, 83]. Therefore, these findings have enabled us to examine the diversity in markers, between the different fractions **(Figure 3.3B)**.

An important thing to be noticed, is that, all the fractions of the USCs derived from controls had particularly exhibited the mesenchymal markers CD73 (98%) and CD105 (78%). On the other hand, we recorded a remarkable decline of the CD73 marker in the fractions F1(82%) and F2(57%) of the USCs derived from DMD patients.

Confronted with USCs derived from DMD patients, the ones from controls, were characterized by the high expression level of the CD105 marker. Conversely, it was perceived that, the distribution of the CD146 marker, a pericyte specific marker, was heterogeneous among fractions of the control USCs (38%, 40%, 50%) respectively for F1, F2 and F3, whereas it was completely absent in DMD USCs fractions.

When we tested the hematopoietic specific markers CD34 and CD45, we observed that neither group had any expression for any of these markers. For all these reasons, our results seem to be consistent with the fact that, the USCs are actually a population of heterogeneous cells.

Additionally, the segregation of cells to unveil the characteristics, can lead to the discovery of further variations in the sub-fractions. In order to reveal the reason behind the variable profiles, that were clearly seen between different cells fractions in both DMD, we proceeded by the study of the dystrophin gene transcript through the use of FluiDMD assay [22].

Results have interestingly shown that, different fractions presented a diversity in the DMD isoforms, this variability was also detected between both DMD (**Figures 3.3 C and D**). The F3 of the DMD with the exon deletion 46-47 has exhibited the expression of the Purkinje (P) full length transcript. Furthermore, this DMD sample is marked by the appearance of the isoform Dp140 that is highly, recognized in the brain and absent in skeletal muscles [84]. As for the muscular (M) and brain (B) full-length isoforms, they were both present in all F2 and F3 fractions.

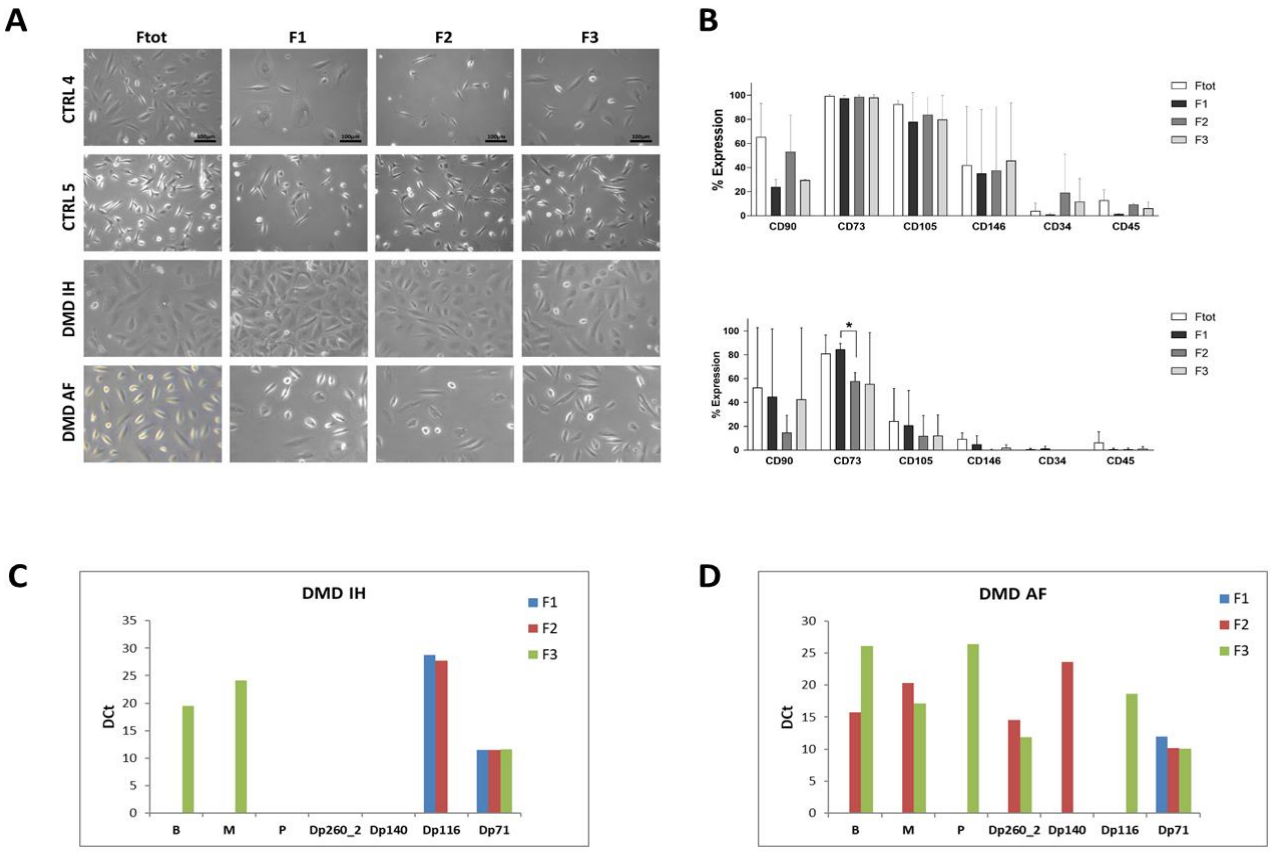

Figure 3.3: Depiction of the variable fractions obtained from controls and DMD USCs.

A) Evaluation of the morphological characteristics of cells in all fractions. Cells were quite similar in terms of morphology in the segregated fractions (F1, F2 and F3) and in the total fraction (Ftot). The usual rounded and spindle -shaped cells were identified precedingly and after sorting by Celector®. The USCs derived from Control 3 were morphologically identical to those obtained in Control 4. **B)** Expression of the different CD markers in control (upper panel), and DMD (lower panel) using the flow cytometry analysis. The graphs (bars) display the mean of the expression of the three different controls (upper panel), and the pair of DMD samples (unpaired t-test: *, $p<0.05$). **C)** FluiDMD or gene microfluidic card shows the different profiling of USCs (native cells) obtained from the DMD IH with a deletion of exons 50-52 versus **D)** DMD AF carrying the deletion of exons 46-47. All DMD different isoforms are amplified by FluiDMD in all the fractions (F1 blue bar, F2 red bar and F3 green bar).

Concerning the F1 fraction that seems to be a bit exceptional when compared to other fractions, we detected the presence of the Dp71 isoform in the variable fractions tested. The latter isoform is characterized by its shortness and omnipresence in many adult tissues with relatively more presence in the central nervous system. Furthermore, the distribution of some other shorter isoforms the Dp260 and Dp116, respectively found in the retina and peripheral nerve, was scattered in the F1 fractions [85].

IV. DISCUSSION

Recently, USCs have drawn much attention and many research papers, have outlined the importance of the usage of USCs, to study a wide variety of diseases, particularly neuromuscular disorders [86] , however, until now, limited information, concerning their characteristics is gathered.

In this study, by using the USCs we attempted to discover the markers, correlated to the DMD disease, through their expression profiling, including the sub-populations characterization.

Our findings, about DMD and healthy controls expression of MyoD-regulated genes, during myogenesis, are consistent with previous results, in different MyoD-induced cells [87]. We also outlined the over-expression of particular markers, implicated in muscle functions, activities and myogenesis. This confirms the previous statement by Falzarano et al, showing that, in order to express the dystrophin protein, USCs should be myogenically transformed [22].

Along with that, it has been disclosed through the GSEA, with the usage of several collections from MsigDB (Hallmarks, Kegg, Reactome, and GO), that following induced myogenesis, deregulation takes place in rare genes, correlated to DMD muscle signature, like inflammation. These gene sets include: granulocyte migration, inflammatory response, interferon alpha response, interferon gamma response, TGF beta signaling and TNFA signaling via NFKb. In addition to that, it has been shown that a partial recapitulation of the muscle DMD phenotype is detected in native USCs.

It is noteworthy to mention that, most of the commonly modulated gene sets in DMD, are even more deregulated also after the USCs myogenesis.

This stresses out that, the DMD muscle features are completely mirrored only in the myogenically transformed USCs, despite the fact that, the native USCs have the ability to express the transcriptional profile of the disease. Thus, we can state that, myogenically transformed USCs are a good model to investigate in depth, the DMD cell characteristics.

Following a gene set enrichment analysis, we have also perceived that, in the DMD derived USCs, gene pathway correlated to mitochondria, is also distorted. In particular,

these genes are for: mitochondrial electron transport NADH to ubiquinone, mitochondrial gene expression, mitochondrial respiratory chain complex assembly, mitochondrial respiratory chain complex IV assembly, mitochondrial translational termination, mitochondrial translation, mitochondrial matrix, mitochondrial translation and oxidative phosphorylation.

This is already recognized, in previous works, where an impairment of the mitochondrial activity, was shown to be present in DMD skeletal muscles [88-90]. Lately, a manuscript has established that, throughout the impaired oxidative phosphorylation, the altered Complex I leads to the increase of mH2O2 [91].

Additionally, in myogenically transformed DMD USCs, we revealed the deregulation of hallmark MYC targets v1 and MYC targets v2 gene sets. The latter targets were newly identified, as directly correlated to tumor severity in breast tumors [92]. Furthermore, it has also been demonstrated, that DMD is associated with cancer progress, the fact which indicates its function in tumor suppression [93].

All these findings, suggest the potential use of USCs, in order to investigate particular cancer circuits in myogenic tumors or even in different types of tumors, involving the modulation of particular dystrophin isoforms of the DMD gene [94-96].

A diversity of the cell type composition was clearly marked, between DMD and healthy USCs, following the deconvolution analysis of RNA-seq data. Thus, we can assume that they have a diverse, expression profile. One of the major properties of USCs, is that they are mesenchymal-like cells, known for their heterogeneity , in terms of cell population, exhibiting diverse expression , morphologic and characteristics in culture [22, 75, 99]. Since little is known about the USCs characteristics, deeper studies are required with the aim of figuring out the possible applications of this new cell model.

The separation method of each sub-population, is hard and inaccurate, since the specific and unique cell surface markers for the identification are absent.

The novel technique Celector® unveiled the presence of no less than three different fractions in both control and DMD USCs. The separation process is established on the principle of cell segregation, according to the physical properties, such as morphology,

size, density and membrane rigidity. Comparing profiles of controls-USCs and DMD-USCs, we observed that, the latter displayed characteristics, that are related to each mutation, while the former were more homogeneous in terms of the CD markers expression as well as for the separation time.

Regarding the expression of the dystrophin isoforms, we noticed that the F2 fraction exhibits the expression of the Dp 140 isoform, which has been known in the literature to be a characteristic of certain areas of the brain in adults. These results, hold great promises for the future studies, because this particular USCs subpopulation, can be the target for the investigation of the specific isoform in the Brain, interpreting the real causes behind the cognitive impairment that affects a remarkable number of DMD boys [100].

It is noteworthy to mention that, the genes upregulation and downregulation shown in the RNA-seq analysis might be correlated to the presence of the different sub-populations of cells varying in size and origin. This can be confirmed by single cell analysis.

Both Celector® and deconvolution analysis, have displayed the variability of expression profile among control and DMD cells suggesting two possible hypotheses: the disease biomarker or a diverse cellular multipotency that can be attributed to cells being in distinct stages of development.

Another important finding to be outlined, is that regardless of the USCs shape (rounded or spindle -shaped), no difference in expression was marked. Indeed, both cell types co-existed in culture after separation.
Consequently, we suggest Celector®, as a very interesting method to separate USCs types and to characterize in depth, these cells to unveil mutations or to examine the expression profile.
The fractions obtained from cell sorting through Celector ® can represent a very good model for cellular immortalization, as well as for iPSCs reprogramming.

An important thing to be outlined, is that Celector ® was not only shown to be efficient in the separation of USCs, but its use also extended to other types of cells and tissues. In fact, it was reported by Rossi et al, in a previous manuscript, that the cell composition of the

stromal vascular fractions, employed for aging related treatments, was successfully examined and characterized following the separation by Celector® [101].

Besides, it has also been demonstrated, that through the use of Celector® technology, human umbilical vein endothelial cells (HUVEC) were subjected to isolation, the fact which is very useful in predicting the diseases that are triggered by abnormalities in the endothelial cells [102].

Furthermore, in a report, Roda and colleagues, have stressed out the importance of this novel technology in sorting the mesenchymal stem cells (MSCs) and their successful separation from the wide variety of possible contamination (.i.e. epithelial cells) ,normally caused by other cell types in a clinical sample [103, 104]. Moreover, in a previously published work, the role of Celector ® was further emphasized in the discrimination of a small population of lymphocytes composed of neoplastic B cells in a specific fraction separated from a mixed population of cells in a blood specimen [105].

In this study, we were the pioneers to emphasize the importance of USCs for RNA profiling, and the recognition of mutations.

To sum up, in order to have a better understanding of the DMD cells, USCs can represent the perfect candidate for this kind of study. In fact, USCs can be very useful in elucidating the gene expression patterns

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List of Acronyms and abbreviations

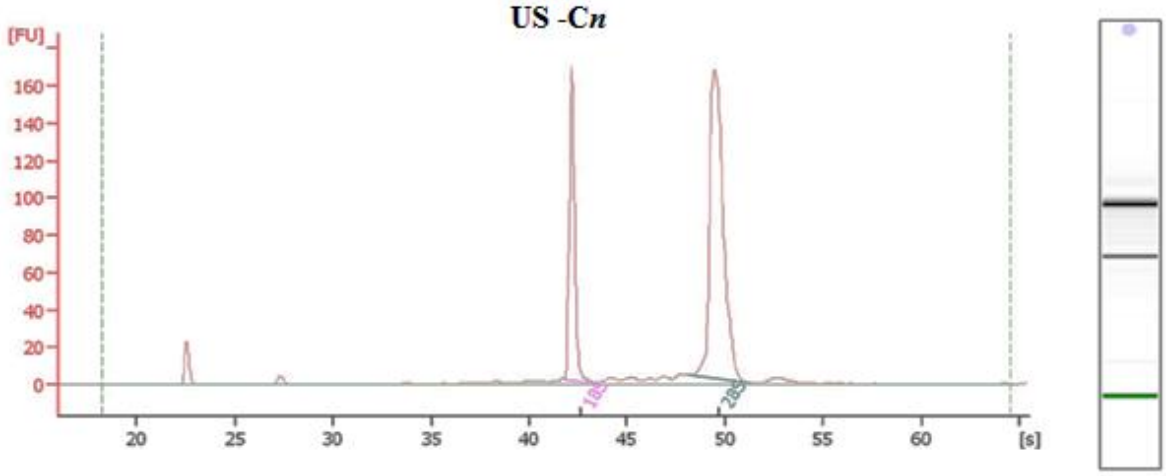
2′OMePS	2′-O-methyl phosphorothioate oligonucleotide
AAV	Adeno Associated Virus
AONs	Antisense Oligonucleotide
BMD	Becker Muscular Dystrophy
CFU-F	Colony -Forming Unit -Fibroblasts
CNV	Copy Number Variation
DGC	dystrophin-glycoprotein complex
DMD	Duchenne Muscular Dystrophy
ECM	Extracellular Matrix
ESCs	Embryonic stem cells
FD	Fabry Disease
GSEA	Gene Set Enrichment Analysis
HLA-DR	Human Leukocyte Antigen – DR isotype
ICST	International Society for Clinical Therapy
iPSCs	Induced Pluripotent stem cells
LGMD	Limb-girdle muscular dystrophy
MSCs	Mesenchymal stem cells
MYOD -1	Myoblast Determination Protein 1
NCAM	Neural cell Adhesion Molecule
NEEGA-DF	Non-Equilibrium, Earth Gravity Assisted Fractionation
NGS	Next Generation Sequencing
PAX2	Paired box genes
PMO	phosphorodiamidate morpholino oligomer
RNA -seq	RNA-Sequencing
RT-PCR	Reverse transcription PCR
SMA	Spinal Muscular atrophy
SMN	Survival motor neuron
UiPSCs	Urine- induced pluripotent stem cells
USCs	Urine-derived Stem cells
XLDC	X-linked Dilated Cardiomyopathy
IFATS	International Federation for Adipose Therapeutics and Sciences
POU5F1	POU domain, class 5, transcription factor 1
OCT3/4	Octamer Binding Transcription Factor
c-MYC	Cellular myelocytomatosis oncogene
SSEA-1/4	Stage Specific Embryo Antigen -1/4
Klf-4	Kruppel-like factor 4
v-WF	Von Willebrand factor

EB	Epidermolysis bullosa
CPM	Counts per million/reads
FDR	false discovery rate
GSEA	Gene Set Enrichment Analysis

Supplementary Materials

The run-on RNA Agilent chip of all samples

USCs pool control

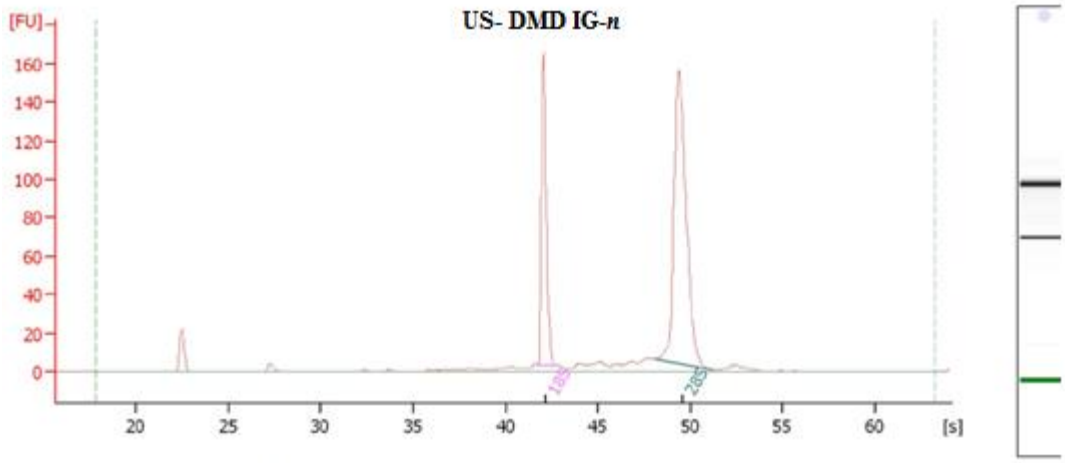


Overall Results for US-Cn

RNA Area:	494,1	RNA Integrity Number (RIN):	10 (B.02.08)
RNA Concentration:	433 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	2,0	Result Flagging Label:	RIN:10

Fragment table for US-Cn

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41,87	43,41	133,8	27,1
28S	48,13	51,36	272,7	55,2

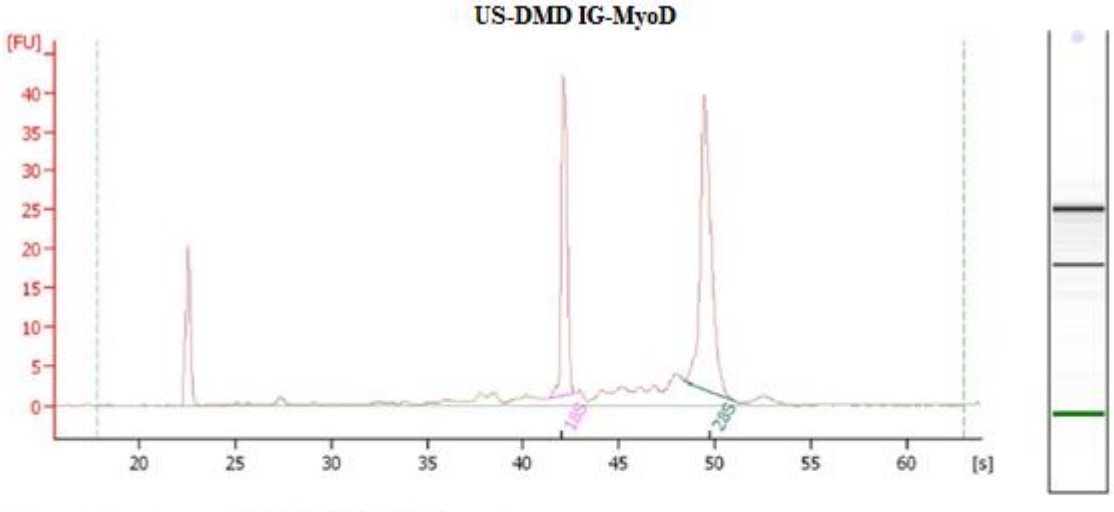


Overall Results for **US-DMD IG-*n***

RNA Area:	474,0	RNA Integrity Number (RIN):	9.9 (B.02.08)
RNA Concentration:	415 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,9	Result Flagging Label:	RIN: 9.90

Fragment table for **US-DMD IG-*n***

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41,75	42,71	124,2	26,2
28S	48,07	51,23	237,2	50,0



Overall Results for **US-DMD IG- MyoD**

RNA Area:	147,8	RNA Integrity Number (RIN):	9.3 (B.02.08)
RNA Concentration:	130 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,6	Result Flagging Label:	RIN: 9.30

Fragment table for **US-DMD IG-MyoD**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41,39	42,72	31,3	21,2
28S	48,38	51,12	50,9	34,4