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MOLECULAR MODIFIERS IN DUCHENNE MUSCULAR DYSTROPHY

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“Non possiamo fare tutto, però dà un senso di liberazione l’iniziarlo. Ci dà la forza di fare qualcosa e di farlo bene. Può rimanere incompleto, però è un inizio, il passo di un cammino.
Una opportunità perché la grazia di Dio entri e faccia il resto. Può darsi che mai vedremo il suo compimento, ma questa è la differenza tra il capomastro e il manovale. Siamo manovali, non capomastri, servitori, non messia. Noi siamo profeti di un futuro che non ci appartiene.”

Oscar Romero (1917-1980)

Vorrei dedicare questa tesi a tutte le persone che in questi anni hanno condiviso con me la fatica e la gioia del lavoro.

Alla Prof.ssa Pegoraro che ha saputo rischiare aprendomi le porte del suo gruppo nel lontano 2013.
A Claudio e Luca con i quali ho condiviso pezzetti del mio lavoro e con i quali sono nate belle amicizie. Che possano continuare nel tempo.
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Ad Aurora e Beatrice, le più giovani e determinate compagne di avventura.
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Summary

Abbreviations

2OMP: 2'-O-methylphosphorothioate

AA: amino acid

AAV: adeno-associated virus

αDG: α-dystroglycan

ARMS-PCR: amplification refractory mutation system polymerase chain reaction method

βDG: β-dystroglycan

BMD: Becker muscular dystrophy

CINRG-DNHS: Cooperative International Neuromuscular Research Group Duchenne Natural History Study

CTGF: connective tissue growth factor

Ctr: control

DAPC: dystrophin associated protein complex

DCM: dilated cardiomyopathy

DFZ: deflazacort

DMD: Duchenne muscular dystrophy

dmm: double-mutant *Spp1* negative mdx mouse

ECM: extracellular matrix

FGF: fibroblast growth factor

HDAC: histone deacetylases

IL1b: interleukin 1b

iOPN: intracellular osteopontin

LoA: loss of ambulation

LD: linkage disequilibrium

LTBP4: Latent Transforming growth factor β Binding Protein 4

MCU: mitochondrial calcium uniporter

mdx: X-linked muscular dystrophy

MMPs: matrix metalloproteinases

MW: molecular weight

NGS: next generation sequencing

NMJ: neuromuscular junction

OPN: osteopontin

ORF: open reading frame

PM: polymyositis

PMOs: phosphorodiamidate morpholino oligomers

PTP: permeability transition pore

SAHA: suberoylanilide hydroxamic acid

SNP: single nucleotide polymorphism

TGF: transforming growth factor

TOMM20: translocase of outer membrane 20

Abstract

Duchenne muscular dystrophy (DMD) is an X-linked progressive neuromuscular disease affecting 1:3500 – 1/5000 boys at birth. It is caused by the absence of dystrophin, a subsarcolemmal protein that confers membrane stability linking cytoskeletal actin to the extracellular matrix. Dystrophin is part of a multi-protein complex called dystrophin associated protein complex (DAPC), which contains, among the other components, β-dystroglycan and nitric oxide synthase (NOS). The consequences of the absence of dystrophin are: deregulation of calcium homeostasis, tissue necrosis, and progressive accumulation of fat and fibrosis and loss of contractile muscle fibers. The ensuing muscle weakness leads to progressive and severe disability, with loss of independent ambulation around the early teens, and cardiac and respiratory failure leading to patient's death, usually around the age of 20-30 years.

Despite all patients having a complete lack of dystrophin in muscle fibers, a relevant inter-patient variability in disease severity is observed (e.g. loss of ambulation may range from 8 to beyond 15 years of age). Emerging evidence points to genetic modifiers, i.e. polymorphisms in genes different form the disease gene, as one of the causes of this variability, but little is yet known about the underlying molecular mechanisms.

My PhD work can be divided into 4 aims:

Aim 1: To characterize the molecular mechanism underlying the modifier effect of the rs28357094 T>G SNP in the *SPP1* gene, encoding osteopontin (OPN) the first identified genetic modifier of DMD. I treated dystrophic and healthy cell line with two different concentrations of deflazacort (DFZ), one of the glucocorticoids mainly used to treat DMD patients, in order to analyze osteopontin expression in relation to genetic background at rs24357094. The results obtained revealed: (I) a developmental regulated expression pattern of OPN; (II) no difference of osteopontin expression are observed related to rs28357094 genotype; (III) an increase in OPN expression only in TG DFZ-treated myotubes, suggesting a possible interaction between glucocorticoid responsive elements (GRs) in the promoter of the *SPP1* gene and the glucocorticoid.

Aim 2: To investigate the possible roles of *SPP1* splicing isoforms in DMD muscle biopsies and cells. Three *SPP1* isoforms, named a, b and c, were analyzed. *SPP1* mRNA studies revealed that all three isoforms are overexpressed in DMD muscle compared to controls, but not in myogenic cell cultures. Moreover, *SPP1* isoforms expression was directly correlated with age in DMD patients' muscle biopsies. Finally, muscle biopsies carrying the rs24357094 TT genotype showed an increased expression of all three *SPP1* isoforms compared to TG genotype.

Aim 3: To validate the known DMD genetic modifiers in novel cohorts of DMD patients utilizing different outcome measures. First, we asked if *SPP1* genotype and *LTBP4* haplotype (the second identified modifier of DMD) can modulate the cardiac involvement in DMD. *LTBP4* haplotye and the *SPP1* rs28357094 were genotyped in 168 DMD patients. *LTBP4* haplotype is composed of 4 polymorphisms in perfect linkage disequilibrium (LD). The genotype at rs10880 resulted, as expected, to be associated to a delay at age of loss of ambulation (LoA) and, as novel finding, also to a delay in cardiomyopathy onset. The *SPP1* minor G allele at rs28357094 resulted also associated to a later cardiomyopathy onset.

Finally, I participate to the identification of the third genetic modifier in DMD: *CD40*. *CD40* was identified through a GWAS approach in a large cohort of DMD patients. The *CD40* rs1883832 C>T polymorphism is located within the Kozak sequence of the gene and it causes a decrease of transcriptional activity of the promoter resulting in an increase of the CD40 secreted isoform. In order to validate CD40 as a genetic modifier in DMD in an independent cohort from the discovery cohort, rs1883832 was genotyped in 96 DMD patients. DMD patients carrying the minor T allele lost ambulation earlier compared to patients carrying the C allele. Moreover, in order to study the functional role of CD40 in DMD, RT-PCR and immunoblot were performed in a subset of patients' muscle biopsies stratified according to rs1883832 genotype. Our results reveal that the minor T allele is associated to an increase of the transcript and a decrease of the protein compared to C genotype.

Taken together these data contribute to clarify some aspects of the molecular mechanisms underlying the downstream consequences of genetic modifiers in DMD. Further studies are needed to fully translate the knowledge acquired in the field of genetic modifiers in DMD to the clinic, e.g. to implement patient genotyping for genetic counseling, prognosis, planning of treatments, and stratification in clinical trials.

Riassunto

La distrofia muscolare di Duchenne (DMD) è una malattia neuromuscolare progressiva legata al cromosoma X con ereditarietà recessiva.

La DMD è causata dalla completa mancanza della distrofina nel muscolo scheletrico. La distrofina è una proteina fondamentale per la stabilità del sarcolemma poiché collega l'actina citoplasmatica alla matrice extracellulare tramite il distroglicano e le proteine associate alla distrofina (*dystrophin associated protein complex*, DAPC). La distrofina ha anche un importante ruolo di signaling e media pathway cellulari implicate nei processi di atrofia/ipertrofia e cascate del segnale legate alla NO sintetasi (NOS). Il deficit di distrofina è dovuto a mutazioni *loss-of-function* a carico del gene *DMD* localizzato in Xp21. Le conseguenze funzionali del deficit di distrofina si traducono, tra le altre, in un'aumentata permeabilità di membrana al calcio con attivazione di proteasi endocellulari e necrosi cellulare. La necrosi cellulare attiva un processo infiammatorio che culmina in una progressiva sostituzione del tessuto muscolare con tessuto adiposo e fibrotico. I pazienti DMD sviluppano precocemente una progressiva debolezza muscolare che li porta alla perdita della deambulazione in un'età usualmente compresa fra i 10 - 15 anni. La prognosi è legata alle complicanze cardiache e respiratorie che sono la causa del decesso tra i 20 ed i 30 anni.

Nonostante tutti i pazienti DMD presentino una completa mancanza di distrofina nel muscolo scheletrico, esiste tra loro un'ampia variabilità nella progressione di malattia in termini di gravità del deficit di forza, età alla perdita della deambulazione (LoA) ed età alla comparsa di insufficienza respiratoria o cardiaca. Recenti studi dimostrano che modificatori genetici (polimorfismi localizzati in geni diversi dal gene *DMD*) hanno un ruolo in questa variabilità, ma il meccanismo attraverso il quale esercitano questa modulazione del fenotipo è ancora poco noto.

Il mio lavoro di tesi riconosce diversi obiettivi:

Obiettivo 1: Il primo modificatore genetico identificato nella DMD è un polimorfismo, rs28357094 T>G, localizzato nel promotore del gene per l'osteopontina, *SPP1.* L'osteopontina è una citochina con un riconosciuto ruolo nell'infiammazione e nel riparo tissutale. L'allele G rs28357094 è stato associato a una più precoce perdita di funzione e/o a una peggiore risposta alla terapia con glucocorticoidi, comunemente utilizzati nella DMD per rallentare la progressione di malattia. Primo obiettivo della mia tesi è stato quello di caratterizzare il possibile ruolo di questo polimorfismo nella DMD. Cellule

muscolari umane derivate da biopsie muscolari diagnostiche di pazienti DMD e controlli sani, accoppiati per età e sesso, sono state trattate con due diverse concentrazioni del glucocorticoide deflazacort (DFZ). Sono stati analizzati sia il trascritto che la proteina nei pazienti e nei controlli stratificati in base al polimorfismo rs28357094. I risultati ottenuti suggeriscono che (I) diverse isoforme di osteopontina siano regolate durante lo sviluppo: una isoforma di circa 50 kDa nei mioblasti e una isoforma di circa 55 kDa nel muscolo maturo. I miotubi esprimono entrambe le isoforme. (II) È stato dimostrato che il genotipo rs284357094 non modifica l'espressione di osteopontina né nei mioblasti né nei miotubi DMD, ma che sia il trascritto *SPP1* che la proteina osteopontina sono upregolati nei DMD rispetto ai controlli. (III) Da ultimo abbiamo investigato quale fosse l'effetto del DFZ sull'espressione dell'osteopontina ed abbiamo osservato che i miotubi TG esprimono più osteopontina quando trattati con DFZ rispetto ai miotubi TT. Questi risultati supportano l'ipotesi che il DFZ interagisca con i *glucocorticoid responsive element* (GRs) localizzati nel promotore del gene *SPP1* e che in presenza del genotipo G rs28357094 la trascrizione di ostepontina sia guidata da GRs e non dai fattori di trascrizione SP1 e SP2 che perdono il loro sito di legame in presenza del polimorfismo.

Obiettivo 2: Il secondo obiettivo di questa tesi è stato lo studio delle diverse isoforme dell'osteopontina, in particolare delle isoforme a, b e c. Sono stati analizzati campioni di biopsie muscolari di pazienti DMD confrontati sia con controlli sani che controlli patologici (polimiosite). (I) I dati ottenuti analizzando i trascritti di tutte e tre le isoforme rivelano che il muscolo DMD esprime più osteopontina (a carico delle tre diverse isoforme) rispetto ai controlli. Nelle colture cellulari non abbiamo osservato diversità di espressione fra DMD e controlli. (II) Stratificando le biopsie DMD per età del paziente al momento del prelievo, abbiamo osservato una diminuzione delle tre isoforme con l'aumentare dell'età. (III) Da ultimo, i pazienti DMD sono stati stratificati in base al genotipo rs28357094 per verificare se il genotipo favorisse l'espressione di una particolare isoforma rispetto alla altre. I pazienti TT tendono ad esprimere rispetto ai pazienti TG una maggior quantità di isoforma a, b e c senza raggiungere la significatività.

Obiettivo 3: Il terzo obiettivo di questa tesi è stato duplice: a) verificare se il genotipo *LTBP4* e *SPP1* abbiano un ruolo nell'esordio della cardiomiopatia nella DMD. b) il ruolo del genotipo CD40 nella patologia DMD.

L'aplotipo IAAM del gene *LTBP4* (Latent Transforming Growth Factor Binding Protein 4), composto da 4 polimorfismi in "*linkage disequilibrium*" (LD) tra loro, è stato associato in maniera significativa ad una più tardiva LoA nei pazienti DMD. Per verificare se i polimorfismi corrispondenti all'aplotipo IAAM e il genotipo rs284357094 del gene *SPP1* correlassero anche con l'età di comparsa della cardiomiopatia nella DMD sono stati genotipizzati 168 pazienti DMD tramite sonde TaqMan. L'età di comparsa di cardiomiopatia è stata definita come l'età di comparsa di una frazione di eiezione del ventricolo sinistro < 50% e/o la comparsa di una dilatazione ventricolare sinistra > 70 ml/mq. Le frequenze alleliche per il gene LTBP4 sono state: rs 2303729(G>A) MAF 39.2%; rs1131620 (A>G) MAF 43.3%; rs10880 (C>T) MAF 39.0%. La frequenza allelica per il gene *SPP1* è risultata per rs28357094 MAF21.1%. Sia l'allele G in rs28357094 *SPP1* che l'allele T in rs10880 *LTBP4* sono risultati significativamente associati con una più tardiva comparsa di cardiomiopatia.

Nel gene di *CD40* è stato studiato un polimorfismo (rs1883832; C>T) localizzato all'interno della sequenza Kozak che ha l'effetto di ridurre il legame del ribosoma al trascritto e ridurre quindi la sintesi proteica. L'allele T in rs1883832 è risultato correlato ad una più precoce età alla perdita della deambulazione in uno studio di associazione con "exome chip" in una coorte di 109 DMD partecipanti allo studio di storia naturale CINRG-DNHS (Cooperative International Neuromuscular Research Group Duchenne Natural History Study). Al fine di validare tale gene come nuovo modificatore genetico nella DMD, sono stati genotipizzati tramite sonda Taqman 96 pazienti DMD, mentre per verificare l'effetto del polimorfismo rs1883832 abbiamo quantificato sia il trascritto che la proteina di CD40 in biopsie muscolari di pazienti DMD. I pazienti sono stati stratificati in base al genotipo rs1883832. I risultati ottenuti mostrano che (I) i pazienti che portano l'allele minore T perdono la deambulazione prima dei pazienti che portano l'allele C; (II) l'allele (T) è associate ad un aumento del trascritto, mentre (III) l'analisi proteica sembra indicare una diminuzione della proteina nei pazienti TT rispetto a quelli CC. Questi risultati sembrerebbero suggerire un effetto dannoso della sovra-regolazione di *CD40*, e sono parzialmente discordanti rispetto agli effetti attesi. Ciò potrebbe sottendere una regolazione complessa dell'espressione di questo gene nel muscolo DMD; sono tuttavia necessari esperimenti su campioni più numerosi prima di trarre conclusioni definitive.

In conclusione, scopo finale della mia tesi di dottorato è stato quello di approfondire il ruolo dei modificatori genetici nella DMD e di studiare il ruolo potenziale di SPP1, LTBP4 e CD40 nell'eziopatogenesi di malattia.

Introduction

Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disease affecting between 1/3500 to 1/5000 (Mendell et al. 2012) live males at birth , caused by the absence of dystrophin, a subsarcolemmal protein, in skeletal muscle Hoffman, Brown, and Kunkel 1987). The absence of dystrophin is due to mutations in the dystrophin gene (*DMD*) located at the Xp21 locus. Dystrophin, a 427 kDa protein, is the central component of the dystrophin-glycoprotein complex that links the actin cytoskeleton to the extracellular matrix, thus maintaining muscle fiber membrane integrity. The lack of dystrophininduces a degeneration of skeletal and cardiac myofibres, as a consequence of mechanical muscle contraction, leading to a dramatic muscle deterioration. Several pathologic phenomena, such as Ca^{2+} leakage, oxidative stress, inflammatory response and fat infiltration, are observed in human dystrophic muscle (Blake et al. 2002). Although the primary genetic defect is known, the downstream consequences of dystrophin deficiency have not been completely understood. Unfortunately, nowadays no definitive curative approaches are available to treat DMD. Oral glucocorticoids (prednisone or deflazacort), respiratory support, cardiological and physical therapy are available to all DMD patients, moreover new emerging therapy aimed to correct the *DMD* gene defect are becoming available. Patients lose the ability to walk independently around 12 years of age, and normally die between the second and third decades due to cardiac or respiratory failure (Lynn et al. 2014).

Dystrophin: from gene to protein

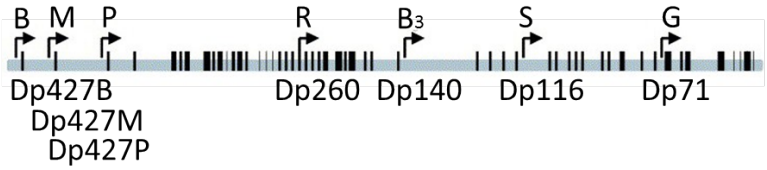
DMD is the largest described protein-coding gene, spanning ~ 2.5 Mb of genomic sequence, and it is composed of 79 exons. The full-length 14 kb mRNA transcribed from the DMD locus was found to be predominantly expressed in skeletal and cardiac muscle with smaller amounts in brain. The protein product encoded by this transcript was named dystrophin since the lack of it causes dystrophy (Hoffman, Brown, and Kunkel 1987).

The expression of the full-length transcript is controlled by three independently regulated promoters situated within a large genomic interval of ~ 400 kb. The brain (B) (Chelly et al. 1990), muscle (M)

(Gilgenkrantz et al. 1992; Klamut et al. 1996; Galvagni, Cartocci, e Oliviero 1998) and Purkinje (P) (Górecki et al. 1992; Holder, Maeda, e Bies 1996; Abdulrazzak et al. 2001) promoters consist of unique first exons spliced to a common set of 78 exons.

The *DMD* gene also has at least four internal promoters that give rise to shorter dystrophin proteins, lacking the actin-binding domain but retaining the cystein rich and carboxy-terminus domains that contain the necessary binding sites for a number of dystrophin-associated proteins. Each of these promoters utilizes a unique first exon that splices into exons 30, 51, 56 and 63, respectively, to generate protein products of 260 kDa (Dp260), highly expressed in the retina, 140 kDa (Dp140), expressed in brain, retina and kidney tissues, 116 kDa (Dp116), only expressed in adult peripheral nerves, and 71 kDa (Dp71), expressed in most non-muscle tissues and is present in cardiac muscle (Muntoni, Torelli, and Ferlini 2003; Doorenweerd et al. 2017) (Fig. 1A and B).

A



B

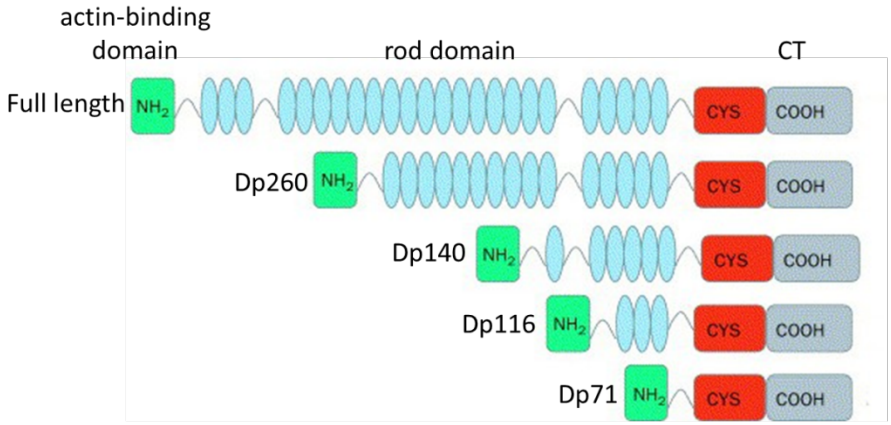


Figure 1: (A) Different promoters in the DMD gene are shown. In a large 5' end genomic interval of 400 kb are situated three promoters that control the expression of the full-length dystrophin (Dp427) in different tissues: B for brain, M for muscle and P for Purkinje. Inside the gene there are also four internal promoters that give rise to the retina (Dp260), brain (Dp140), peripheral nerves (Dp116) and general ubiquitously expressed (Dp71) isoforms of the protein. (B) Schematic representation of dystrophin protein and its isoforms. The full-length protein is organized into four separate domains:

the actin-binding domain at the NH2 terminus, the central rod domain, the cysteine-rich domain and the COOH-terminal domain. Each of the isoforms maintained the N- and C- terminus domains and the CYS domain, while the rod domain varies in length. Adapted from (Muntoni et al. 2003).

The molecular weight of the full length dystrophin is 427 kDa. Dystrophin is linked to the sarcolemma of normal muscle by a protein complex composed of at least 10 different proteins: the dystrophin-associated protein complex (DAPC) (Figure 2). This complex spans the membrane and links the actin-based cytoskeleton to the muscle basal lamina. Thus the DAPC can be thought of as a scaffold connecting the inside of a muscle fiber to the outside. The DAPC can be divided into several separate subcomplexes based on their location within the cell and their physical association with each other, the dystroglycan complex, the sarcoglycan/sarcospan complex and the cytoplasmic dystrophin-containing complex (Hoffman et al. 1987).

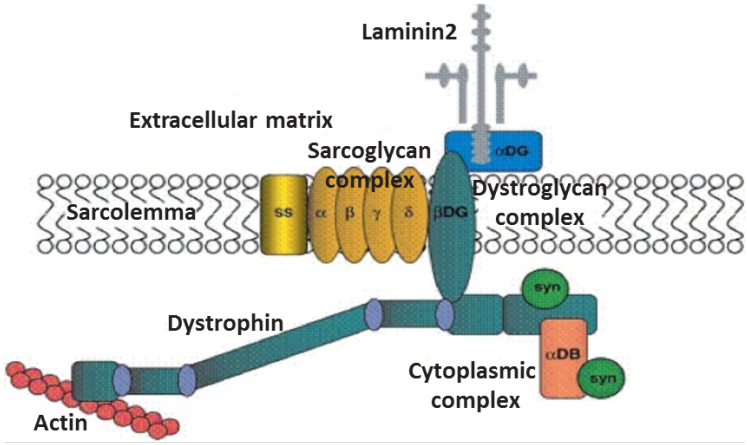


Figure 2: The dystrophin-associated protein complex (DPC) in skeletal muscle. Dystrophin binds to cytoskeletal actin at its NH2 terminus. At its COOH terminus, dystrophin is associated with a number of integral and peripheral membrane proteins that can be classified as the dystroglycan subcomplex, the sarcoglycan-sarcospan subcomplex, and the cytoplasmic subcomplex. The cytoplasmic subcomplex includes the syntrophins and α-dystrobrevin (αDB). The sarcoglycan-sarcospan subcomplex comprises the sarcoglycans (α, β, δ, γ) and sarcospan. The extracellular component of the dystroglycan complex, α-dystroglycan (αDG), binds to laminin-2 in the extracellular matrix and β-dystroglycan (βDG) in the sarcolemma. In turn, β-dystroglycan binds to dystrophin, thus completing the link between the actin- based cytoskeleton and the extracellular matrix. Adapted from (Blake et al. 2002).

Gene mutations

In DMD patients, the open reading frame of the dystrophin gene is altered by deletions, duplications, point mutations, or other rearrangements (Kalman et al. 2011). Being an X-linked disorder, DMD almost exclusively affects males, who are usually born from asymptomatic carrier mothers. However, a significant number (around 30%) of all DMD cases are the result of *de novo DMD* gene mutations that can occur in the mother's germinal line regardless of family history (Bennett et al., 2001). The majority of large deletions cluster around two mutational hot spots, probably due to the chromatin structure in the Xp21 region. Region I spans exons 45-53 and contains exons coding for part of the rod domain, while region II spans exons 2-20 and contains exons coding for some or all of the actin-binding sites together with part of the rod domain (Blake et al. 2002). Thanks to Monaco, a model of correlation between a *DMD* gene deletion and the severity of the phenotype has been established (Monaco et al. 1988). Mutations that maintain the reading frame of the *DMD* gene, resulting in partially functional protein, are associated to the Becker muscular dystrophy (BMD) phenotype, where mutations that alter the reading frame of the gene, resulting in unstable mRNA that eventually leads to the production of nearly undetectable amounts of truncated protein, are associated to the DMD phenotype (Figure 3). This theory is valid in 90% of the cases but some exceptions have been described (Koenig et al. 1989; Malhotra et al. 1988; Winnard et al. 1993; Kesari et al. 2008; Muntoni et al. 1994; Winnard et al. 1995; Gualandi et al. 2006; van den Bergen et al. 2014; Pane et al. 2014; Aartsma-Rus et al. 2006).

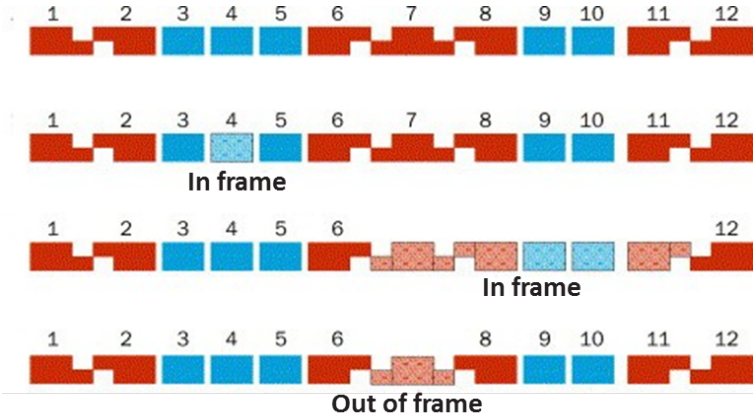


Figure 3: Schematic representation of in frame and out of frame deletions. (A) The open reading Frame (ORF) is maintained with exon 4 or exon 7-11 deletion. On the other hand, the ORF is lost when exon 7 is deleted. Adapted from (Muntoni et al. 2003).

In around 10-15% of DMD patients point mutations have been described. Point mutations do not show mutational hotspot, and can be identified along the entire sequence of the gene. Nonsense mutations introduce a premature stop codons and usually very little or no protein is detected (despite the hypothesis of the production of normal amount of truncated protein) (Blake et al. 2002). Small, frameshifting insertion-deletions of a few nucleotides and splicing mutations are very rare (Kilimann et al. 1992; Winnard et al. 1992).

Pathophysiology

The complete absence of dystrophin has profound functional and structural consequences in muscles: alterations of membrane permeability, intra-cellular calcium homeostasis, muscle strength, and muscle regeneration capacity (Blake et al. 2002).

Membrane permeability

The first sign of a malfunction of the membrane permeability is the presence of muscle enzymes found in the serum of dystrophic patients. In 1959 Ebashi et al. showed for the first time a systematic increase of CK activity in patients with progressive muscular dystrophy. Later, Okinaka and Schapira observed very high CK level in DMD patients and in about 2/3 of the patients' mothers (Okinaka and Schapira1961). HyperCKemia is the result of a fragile of the lipid bilayer in dystrophic muscles (Ozawa et al. 1999).

Even if it is an important biomarker for DMD diagnosis, CK level varies during life and with exercise. It is maximal between 1 and 6 years and then declines (Zatz et al. 1978; Govoni et al. 2013). Some other muscular enzymes can be detected in the serum of muscular dystrophy patients, for example pyruvate kinase, parvalbumin or myoglobin (Jockusch et al. 1990; Kagen et al. 1980; Zatz et al. 1978). Some extra-muscular molecules can be detected in muscles, for example albumin, different IgGs and IgMs (Straub et al. 1997). Membrane permeability can be studied in *in vivo* experimental model through the use of colored vital markers that are able to pass the plasma membrane, such as Evans Blue which links albumin, and is used to detect necrotic fibers. Permeability differs between different muscles, and there is a certain variability in the degree of necrosis between fiber types within the same muscle, type II (glycolytic) fibers being more prone to undergo necrosis (Straub et al. 1997).

Calcium deregulation and mitochondria implication

Ca^{2+} deregulation has been implicated in muscular dystrophy (Deconinck et al. 2007). In particular, the lack of dystrophin is believed to lead to increases in Ca^{2+} permeability into muscle fibers through different mechanisms, including membrane tears and channel-mediated ion fluxes et al. 2011; Péréonet al. 2015; Blat and Blat 2015). Since mitochondria act as a low affinity/high capacity Ca^{2+} buffer system, the increase in cytosolic Ca^{2+} concentration occurring in DMD patient might correspond to an increase in mitochondrial Ca^{2+} accumulation, leading to mitochondrial Ca^{2+} overload and cell death (Deconinck and Dan 2007). Supporting this model, the reduction of mitochondrial swelling and necrotic tissue in *mdx* were observed after cyclophilin inhibitor treatment (Millay et al. 2008).

Inflammation and fibrosis

Dystrophin-deficient skeletal muscles and hearts show some degree of inflammation, and inflammatory cells are known to promote fibrosis in numerous pathologies (Strutz and Neilson 2003). In the scenario of a muscular dystrophy inflammatory cells may induce fibrosis by secreting cytokines such as transforming growth factor (TGF), which stimulate connective tissue production by fibroblasts (Gosselin et al. 2004). Moreover, previous studies et al. 2001) showed that inflammation in dystrophic skeletal muscles is exacerbated by the loss of nNOS from muscle. As a consequence, the expression of an nNOS transgene in *mdx* skeletal muscles can normalize NO production and cause a reduction of the inflammation.

The inflammatory process in muscle can be divided into two different stages: an early inflammatory phase, dominated by the activation of the transcription factor NF-κB and its transcriptional targets, and a chronic phase in which TGFβ plays a pivotal role (Chen et al. 2005). In DMD muscle biopsies a secondary inflammatory cell infiltrate is observed, especially close to foci of degeneration and necrosis, predominantly constituted by macrophages, T lymphocytes and neutrophils. Not only these cells, but also muscle cells themselves, actively secrete a wide array of pro-inflammatory cytokines (Engel and Arahata 1986; Shin et al. 2013).

Replacement of muscle tissue by collagen and fatty deposits is a common feature in DMD patients and *mdx* mice, and increase with age (Goldspink et al. 1994) partially due to the loss of proliferative capacity of muscle satellite cells, which hinders regeneration and repair (Pastoret and Sebille 1995, 1993). The accumulation of collagen (especially type III) probably causes further membrane damage during contractile activity, in a vicious cycle leading to further collagen accumulation. This was

observed in *mdx* diaphragm, a muscle whose activity is constantly requested (Goldspink et al. 1994). Furthermore, a progression of collagen deposition is observed throughout life: for example at 9 months of age *mdx* mice present 4 times more collagen than wild type mice at the same age, and, at 12 months, 7 times more (Itagaki et al. 1995).

In DMD patients, transforming growth factor (TGF)-β l, which is the best-characterized fibrogenic mediator, is overexpressed (Heydemann et al. 2009) and the increase of its mRNA level is associated with the initial stage of tissue fibrosis. Subsequently, connective tissue growth factor (CTGF), the downstream autocrine mediator of TGF-β l, is overexpressed (Igarashi et al. 1993). CTGF is an extracellular matrix-associated protein expressed at high levels in fibroblasts that modulates many cellular functions, including proliferation, migration, adhesion and extracellular matrix deposition. In addition, being a potent fibroblast mitogen and chemoattractant, CTGF stimulates the production of procollagen and fibronectin in fibroblasts (Sun et al. 2008). TGF-β is able to promote fibrosis through the activation of IL4, 5 and 13 (Zhou and Lu 2010), and osteopontin (Zanotti et al. 2011; Vetrone et al. 2009). TGF- β is secreted as part of a latent complex in association with latent TGF-β binding proteins (LTBPs). The cleavage of the latent binding protein by proteases such as matrix metalloproteases (MMPs) is necessary for TGF-β release and interaction with its receptors located on the cell surface.

Mezzano et al. have provided evidence of a particular role of TGF-β 1 and CTGF in fibroblasts isolated from the diaphragm of adult *mdx* mice. In particular, TGF-β 1 and CTGF are able to control the levels of fibronectin (FN), an extracellular matrix protein. Moreover, they speculate that dystrophic fibroblasts are "reprogrammed" during disease course, as they did not find the same pattern of FN expression in fibroblasts isolated from the diaphragm of young *mdx* mice, compared with old mice. Furthermore, when wild type fibroblasts are incubated with medium of *mdx* fibroblasts, they produce more FN protein that in normal conditions. This suggests that dystrophic fibroblasts secrete into their culture medium some factors that can stimulate extracellular matrix synthesis, thus maintaining a fibrotic phenotype (Mezzano et al. 2007).

Perturbation of NO synthase

NO is generated by NOSs, a group of conserved cytosolic or membrane-bound isoenzymes.

The three mammalian NOS enzymes are coded by three different genes with a similar genomic structure: neuronal (nNOS, type I), endothelial (eNOS, type III) and inducible (iNOS, type II). In healthy

adult mammals, the muscle isoform (muNOS) is localized under the sarcolemma. This alternative spliced isoform, with catalytic activity equivalent to the neuronal isoform, has an insertion of 102 base pairs between exons 16 and 17, corresponding to 34 amino acids (AA) (Silvagno et al. 1996). In DMD patients and in *mdx* mice the muNOS is not anchored at the sarcolemma because of the absence of dystrophin, but it is localized in the cytoplasm and is expressed at a lower level compared to control samples (Brenman and Bredt 1997; Chang et al. 1996). The reduction of NO production by NOS may leads to ischemic phenomena due to insufficient vasodilation (Péréonet al. 2015; Bello et al. 2013).

Oxidative stress

An increase of reactive oxygene species (ROS) has been described in dystrophic muscle (Spurney 2011; Whitehead et al. 2010). It is not fully understood why the absence of dystrophin leads to ROS overproduction (Rando 2002), but calcium deregulation and inflammation might promote an increase of ROS through mitochondria (Terrill et al. 2013). Moreover the absence of dystrophin leads myotubes to be more susceptible to oxidative injury than wild type myotubes (Rando et al. 1998). An increase of sarcolemmal damage and antioxidant gene expression have been observed (Disatnik et al. 1998).

Clinical features of DMD

High serum CK and muscle fiber necrosis are present in the neonatal period, but symptoms are seldom manifested during the first 2 years of life. Early symptoms are developmental delay, difficulty in running or climbing stairs, frequent falls and enlargement of the calf muscles. Between 3 and 6 years of age, waddling gait, hyperlordosis and Gowers sign appear.

Age at loss ambulation (LoA) is considered a milestone in the natural history of DMD, and it occurs before the age of 13 according to a classic clinical definition. This milestone has been postponed by improved standards of care and glucocorticoid treatment (McDonald et al. 2018). Recent works underline that the age at LoA is influenced not only by external factors, such as steroid treatment, surgical treatment, excessive weight gain, but also by genetic modifiers (Pegoraro et al. 2011; Bello et al. 2015).

A progressive loss of strength in diaphragm and heart leads to the onset of respiratory insufficiency, eventually requiring mechanical ventilation, and to cardiomyopathy. An exacerbation of respiratory dysfunction in affected boys is due to progressive kyphosis (Velasco et al. 2007). As described for LoA, respiratory insufficiency could be delayed with steroid treatment (Daftary et al. 2007).

Dilated cardiomyopathy onset is variable but by the age of 18, the majority of patients exhibit clinical signs of cardiac involvement (Nigro et al. 1990). Left ventricular wall abnormalities and left ventricular dilation have been identified as common pattern of myocardial dysfunction in DMD patients (Melacini et al. 1996)

Clinical variability and genetic modifiers

It is well known that in Duchenne muscular dystrophy, *DMD* gene mutations result in a complete absence of dystrophin in patients' skeletal muscle. Even if DMD patients are biochemically equal (all share dystrophin deficiency), a substantial variability in disease onset, progression and response to

therapy have been reported (Paneet al. 2014; Henricson et al. 2013; McDonald et al. 2013) Recent studies have shown the importance of genetic modifiers in DMD progression. A genetic modifier is a common or rare variant (SNP, single nucleotide polymorphism) in a gene unrelated to the primary gene defect, able to modulate disease progression. Our group identified the first genetic modifier in DMD :a polymorphism at position -66 bp in the promoter region of *SPP1* (rs28357094) (Pegoraro et al, 2011). rs28357094 is predicted to interfere with SP1 binding site and to result in a decrease osteopontin (OPN) expression (Giacopelli et al. 2004) The G allele at rs28357094 was found to be associated to an earlier age at LoAin an Italian cohort, and to a decreased grip strength in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS) (Pegoraro et al. 2011). rs28357094 was later validated in a second, independent, Italian cohort (Bello et al. 2012).

A second modifier was recently identified with a novel approach. A deletion/insertion of 36 bp in the *ltbp4* gene was identified by genome-wide mapping in a sarcoglycan deficient murine model (Heydemann et al. 2009). Ltbp4 encodes a latent TGF-beta-binding protein that sequesters TGF-beta and regulates its availability for binding to the TGF-beta receptor. Insertion of 12 amino acids into the proline-rich region of LTBP4 reduced proteolytic cleavage and was associated with reduced TGF-beta signaling, decreased fibrosis, and improved muscle pathology in a mouse model of muscular dystrophy (Heydemann et al. 2009). Based on this findings *LTBP4* become a candidate as genetic modifier in muscular dystrophy. Flanigan et al in 2013 identified in the human homologue, *LTBP4* gene, a complex haplotype resulting from 4 SNPs in LD. They showed that IAAM haplotye was associate with a later age at LoA in the United Dystrophinopathy Project (UDP) cohort of around 254 DMD patients (Flanigan et al, 2013). This association was then validated in the CINRG-DNHS cohort (Bello et al. 2015).

The study of genetic modifiers could help to understand the basis of phenotypic variability in DMD patients and could point at novel therapeutic targets, improve prognosis and personalization of treatments and will allow a more accurate design and analysis of clinical trials. However, a detailed characterization of the molecular mechanisms underlying these association is needed, in order to translate them to a use for patient care.

Therapies and trials

Therapeutics approaches to treat DMD can be separated into two different categories: palliative treatments, such as drugs targeted to the pathologic consequences of dystrophin deficiencies, and aetiologic treatments targeted to restore the gene or protein, such as cell and gene therapies.

Currently glucocorticoids are gold standard and the most diffusely prescribed treatment for all DMD patients regardless of their *DMD* gene mutations (Matthews et al. 2016; Gloss et al. 2016; Koeks et al. 2017), . Among steroids, deflazacort (DFZ) is emerging as potentially more effective (Bello et al. 2015) and more tolerable than prednisone (Bonifati et al. 2000; Griggs et al. 2016), but with both these drugs response to treatment is variable, some patients showing greater clinical benefit than others. Glucocorticoids are typically used to suppress inflammation as they enhance the transcription of anti-inflammatory genes, and inhibit pro-inflammatory factors (Baltgalvis et al. 2009). In addition, part of the benefits observed with glucocorticoids could be attributed to their capacity to enhance utrophin expression (Miura et al. 2008).

As we write, an unprecedented number of clinical trials are ongoing on to test new approaches to treat DMD.

Some example of clinical trial undergoing (update 2017).

- STG-001 (started in Dec. 2017): based on microdystrophin expression in target organs through an AAV vector (Karin 2017). In order to address the expression of the minigene, a tissue specific promoter for skeletal muscle, diaphragm and heart is added. The powerful aspect of gene therapy is its independence from any particular mutation in DMD gene.
- SMT C1100 (Ezutromid. Clinical phase 2): the target of this approach is utrophin, the foetal homologue to dystrophin. Utrophin is the foetal homologue of dystrophin sharing 80% AA) identity. The comparison between the two AA sequences showed that both proteins share the same actin binding domain, rod domain, CYS domain and C-terminus domain. The expression of utrophin has been studied during human development in healthy and dystrophic conditions. It is present in the muscle before the 9^{th} week of gestation with a non-homogeneous pattern; then protein expression becomes uniform and maximal expression can be observed at 17-18 weeks. At the 26^{th} week of gestation the expression decreases, and the localization is the same as in adult fibers (Clerk et al. 1993). In adult healthy muscle the gene of utrophin remains intact but quiescent and the protein is confined at the NMJ and myotendinous junctions (Lin and Burgunder 2000; Blake et al. 2002). Like the *DMD* gene, the utrophin gene is under the control of different promoters that allow the expression of the full length (A and B promoters) or of the truncated isoforms (Up71, Up 113 and Up140).

- Exon skipping approach: the aim of this approach is to convert the severe form of the disease into a milder form modulating pre-mRNA splicing and achieving reading frame restoration (Aartsma-Rus 2012; Fairclough, Perkins, and Davies 2012) thanks to antisense oligonucleotides (AONs), mainly phosphorodiamidate morpholino oligomers (PMO). Clinical trials undergoing target exon 53 (SRP-4053, phase III. NS-065/NCNP-01), exon 45 (SRP-4045, phase III) and exon 51 (Eteplirsen also known as ExonDys 51, NCT02255552, phase III). For more details see («Clinical Trials | Sarepta Therapeutics» s.d.). Based on results obtained with the prolonged phase 2b extended study, the Food and Drugs Administration has granted accelerated approval to Eteplirsen in the United State of America (Mendell et al. 2016). The development program of AONs based on the 2'-O-methyl phosphorothioate backbone was interrupted due to an insufficient efficacy/toxicity ratio in phase 2/3 clinical trials (Goemans et al. 2018).
- The absence of dystrophin in about 10-15% of DMD patients is due to nonsense mutations, that convert an amino acid into a premature stop codon (Pichavant et al. 2011). Translarna (phase III), also known as Ataluren or PTC124, like gentamicin, an aminoglycoside antibiotic, is able to enable stop codon read-through by introducing a cognate aa at the premature stop codon to continue the mRNA translation («PTC Therapeutics | Clinical Trials: Ataluren» s.d.). This phenomenon is known as "stop codon read-through" (Pichavant et al. 2011). Ataluren has been approved by the European Medicine Agency thanks to the efficacy in the phase 2b (Bushby et al. 2014).
- Mitochondrial target: (+)-Epicathenin (phase I/II) and Catena®/Raxone® (idebenone). Recent studies on idebenone have shown an improvement of respiratory capacity on DMD patients (Buyse et al. 2017).
- Steroid treatment refinement and steroid replacements: as mention above steroid treatment is the main treatment available for DMD patients, but side effects are often present. In order to minimize the side effect different studies are ongoing. FOR-DMD (phase III) compares deflazacort and prednisone regimens and VBP15-004 (phase IIb) studies the effect of a new "dissociative" steroid, vamorolone, in DMD (for more information see http://www.reveragen.com/vamorolone).
- Inflammation and fibrosis target: continues cycles of degeneration and regeneration take place in the muscle of DMD patients, with subsequent inflammatory processes which initially stimulate repair, but eventually lead to fibrosis, until muscle is replaced by fibrotic tissue.

Edasalonexent (phase I/II) is a small molecule able to inhibit NF-kB, known to drive muscle inflammation and degeneration (Donovan et al. 2017) while FG-3019 (phase II) is a monoclonal antibody against the connective tissue growth factor (CTGF).

- HDAC (histone deacetylases) target: from the beginning of 2000, HDAC inhibitors such as givinostat and suberoylanilide hydroxamic acid (SAHA) became attractive molecules for DMD treatment (Colussi et al. 2010; Consalvi et al. 2013). Consalvi et al. observed *in vitro* an increase of the size of the human myotubes and, *in vivo*, in the animal model of DMD, *mdx* mice, an increase of muscle mass accompanied by an increase of muscle fiber cross sectional area , a drastic reduction of fibrosis and fatty infiltration and a reduction of MPO activity (a marker of inflammation) after givinostat treatment. They also observed an increase of functional parameters of skeletal muscles activity and a reduction of Evan's blue dye uptake. Currently, Givinostat is undergoing a phase III clinical trial. Colussi et al. treated *mdx* mice with SAHA and described the recovery in muscular strength and histological amelioration, accompanied by the rescue of plasma protein profile. In particular they observed an increase in muscle cross sectional area, and a decrease of inflammation and fibrosis.
- Myostatin target: Myostatin is a well-known negative regulator of muscle growth. Molecules currently under study are Domagrozumab/PF-06252616 (phase II) and BMS-986089 (phase II/III).
- Other target: Rimeporide (phase I) against sodium and calcium overload. Eplerenone and Spironolactone (phase III) protect the myocardium in association with other commonly used cardiac drug such as angiotensin converting enzyme inhibitors and β-blockers.
- Finally, CAP-1002 (phase I/II) is an innovative cell strategy based on cardiac stem cells that are able to secrete factors promoting cardiac regeneration, inhibiting inflammation, fibrosis and oxidative stress (Chakravarty et al. 2017).

Aims

Despite the molecular bases of DMD have been known for 30 years, effective treatments remain elusive and many aspects of the pathophysiology are yet not clear. The observation of the relevant clinical variability between DMD individuals, and the discovery of genetic modifier loci, have suggested that genes and proteins implicated in the pathophysiology of muscle damage and repair

may dictate the severity of the phenotype, and represent potential therapeutic targets. When modifier loci are identified through association studies, “wet lab” experiments are necessary to confirm and characterize the biological mechanism underlying the genotype-phenotype association. In particular, in this thesis we aimed to characterize several mechanistic aspects of some known modifier loci, such as *SPP1*, *LTBP4* and *CD40*.

Aim 1. Does the rs28357094 SNP regulate *SPP1* expression in myogenic cells? The minor allele at this SNP was shown to be associated with more severe phenotypes, and/or to reduced response to glucocorticoids (Pegoraro et al. 2011; Bello et al. 2015). We used primary myoblast and myotube cultures treated with DFZ to explore the expression of *SPP1* transcript and its OPN protein product. As the SNP is situated in the *SPP1* promoter, which contains glucocorticoid responsive elements, we hypothesize that rs28357094 genotype modifies transcript/protein expression at baseline and in response to glucocorticoids.

Aim 2. Do *SPP1* splicing isoforms have a role in the modifier effect of this locus in DMD? *SPP1* is known to be alternatively spliced into 3 major isoforms which undergo different post-translational modifications, and which have specific biological roles in other diseases (e.g. cancer). However, little is known about the expression of these isoform in muscle health and disease. We set to test the hypothesis of a correlation between differential expression of these isoforms with rs28357094 genotype and clinical DMD features in muscle biopsy samples and primary muscle cell cultures.

Aim 3 focuses on aspects of the validation of genetic modifiers in DMD. In particular, we asked if *SPP1* genotype and *LTBP4* haplotype are also related to the cardiomyopathy phenotype. The last modifier, identified thanks to a GWAS approach, is *CD40*. We validated the gene in the Padova cohort through the genotyping of 96 DMD patients. Moreover, in order to study the functional role of this gene in DMD, RT-PCR and immunoblot have been performed in muscle biopsy samples.

Aim 1: Does the rs28357094 SNP regulate *SPP1* expression in myogenic cells?

***SPP1* (rs28357094). From "*SPP1* genotype and glucocorticoid treatment modify osteopontin expression in Duchenne muscular dystrophy cells".**

OPN is a member of the small integrin-binding N-linked glycoprotein family (Denhardt et al. 2001) first identified as an adhesive component of the extracellular matrix of the bone tissue. OPN is expressed in a wide range of cells, including epithelial cells, osteoblasts, osteoclasts, immune cells (especially macrophages and lymphocytes), and muscle cells. It is transcribed in five different isoforms due to alternative splicing and can be secreted as a soluble cytokine or as a component of the extracellular matrix (ECM), while an intracellular form of the protein (iOPN) has been described in murine cells (Shinohara et al. 2008; Gimba et al. 2013). OPN is extensively modified by glycosylation, phosphorylation, sulphation (Gimba et al. 2013), and proteolytic cleavages by thrombin and matrix metalloproteinases (MMPs) (Yokosaki et al. 1999), thus resulting in a complex variety of functionally distinct units. OPN is involved in a number of biological processes that include tissue repair, cancer, inflammation, and fibrosis (Berman et al. 2004; Giachelli et al. 1998; Hashimoto et al. 2006; Liaw et al. 1994; Mori et al. 2008; O'Regan and Berman 2000).

In the context of muscle pathology, data from the animal models of DMD seem to be discordant. The ablation of *SPP1* was shown to have beneficial effects in the standard *mdx* mouse; specifically, the double-mutant *Spp1* negative mdx mouse (*dmm*) showed a diminished amount of intramuscular neutrophils and NKT-like cells, resulting in a decrease of TGFß-mediated signalling and fibrosis (Vetrone et al. 2009). The same laboratory demonstrated a shift to a pro-regenerative macrophage phenotype in the *dmm* model (Capote et al. 2016). On the other hand, very recently Calyjut et al. demonstrated that when transferring the *mdx* mutation to a different mouse strain, the new *mdx*$^{129/Sv}$ animals showed a substantially milder phenotype than standard *mdx*, even though the former express *Spp1* at markedly higher levels than the latter (Calyjur et al. 2016). Despite the controversies on the beneficial or harmful effect of OPN in muscle dystropathology, it is clear that the *Spp1* gene expression is strongly induced (up to 100-fold) after acute muscle damage (Hoffman et al. 2013a), and that OPN plays a relevant role both as a cytokine, by recruiting inflammatory cells (Uaesoontrachoon et al. 2013), and as a structural component of the nascent extracellular matrix surrounding regenerating myotubes (Pagel et al. 2014; Uaesoontrachoon et al. 2008).

A single nucleotide polymorphism (SNP), 66 bp upstream the SPP1 starting codon (rs28357094), has been identified as a genetic modifier of weakness progression in DMD (Pegoraro et al. 2011) and muscle size in healthy females (Hoffman et al. 2013a).

In particular, DMD patients carrying the G allele (TG or GG) showed reduced grip strength and earlier age at loss of ambulation (LoA) (Bello et al. 2015; Pegoraro et al. 2011) compared to patients homozygous for the T allele (TT). This association was validated in an independent DMD cohort, in which the G allele was associated to a more rapid decrease in the 6 minute walk test performance (Bello et al. 2012), but could not be reproduced in two other independent DMD cohorts (van den Bergen et al. 2014; Flanigan et al. 2013). rs28357094 was shown to modulate OPN transcription efficiency in HeLa and muscle cells, the G allele being associated to reduced expression (Pagel et al. 2014; Uaesoontrachoon et al. 2008), but the rs28357094 genotype did not dictate any difference in SPP1 mRNA or OPN protein levels in DMD muscle biopsies (Piva et al. 2012). Thus, a discrepancy is evident between the mdx mice and human, as mice lacking OPN perform better and DMD boys carrying the G allele (predicted to drive a less efficient SPP1 transcription (Uaesoontrachoon et al. 2008), are weaker.

A recent study, aiming to validate the association of the rs28357094 G allele with earlier loss of ambulation in DMD, showed that the detrimental effect of this genotype was only observed in glucocorticoid-treated DMD patients (Bello et al. 2015). These clinical studies, together with evidence that the SPP1 promoter can be modulated by steroid hormones (Barfield et al. 2014), suggest the hypothesis that rs28357094 might be a pharmaco-dynamic biomarker of glucocorticoid response in DMD, rather than a predictor of disease progression *per se*.

Based on this evidence, to further elucidate the role of rs28357094 genotype on OPN expression, we investigated OPN expression in skeletal muscle myoblasts and myotubes obtained from DMD patients and healthy controls with defined rs28357094 genotype. Moreover, we studied the effect of the glucocorticoid deflazacort (DFZ) on OPN expression in different rs28357094 genotypes, to verify the hypothesis that steroid responsive elements in the SPP1 promoter region may predict response to glucocorticoid treatment.

Materials and Methods

Ethics approval:

Muscle biopsies were collected from patients according to the requirements of our Institutional Ethical Committee and of the Helsinki Declaration of 1975, as revised in 1983.

Patients:

Eleven subjects were selected from a cohort of DMD patients followed at the Neuromuscular Center of the University of Padova. All patients showed total absence of dystrophin in skeletal muscle and/or out-of-frame or nonsense *DMD* mutations. Mean age at biopsy ± standard deviation was 3.7 ± 2.3 years. DNA samples were obtained after informed consent. Nine normal pediatric muscle biopsies were used as controls (mean age at biopsy ± standard deviation 6.8 ± 5.1 years). Patients' muscle biopsies were obtained at the time of diagnosis, prior to any steroid treatment.

Cell isolation, culture and differentiation:

Human muscle biopsies from patients and healthy controls were collected, minced into small pieces and placed in a solution of 0.8% w/v collagenase I (Life Technologies; Carlsbad, CA, USA) in DMEM, supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Life Technologies; Carlsbad, CA, USA), for 60 minutes. After digestion, muscle fragments were gently dissociated by pipetting with a 2 ml and 1 ml pipette, before being passed through a 21 G syringe needle for 20-25 times. Two volumes of growth medium (see below) were added to the digestion mix and the resulting cell suspension was centrifuged for 10' at 300g . The resulting pellet was eventually resuspended and plated on a matrigel-coated 35 mm well in growth medium composed of 20% FBS, 25 ng/µl hFGFb (human Basic Fibroblast Growth Factor, Immunotools; Friesoythe, Germany) in Ham's F12 medium (Euroclone; Milan, Italy) with Pen/Strep. Cells were expanded for 2-3 passages in 60 and 100 mm dishes. Examples of the cultures are shown in Supplementary Figure 2.

Isolation of $CD56^+$ cells:

Cell cultures obtained by enzymatic and mechanical disruption of muscle fragments were further enriched in CD56 positive cells (CD56+), as a *bona fide* marker of myogenic cells. Cells were detached in citrate buffer (14.5 mM trisodium citrate, 134 mM KCl) in order to preserve the surface proteins, collected, bound to the CD56 MACS microbeads and separated with the MACS Columns (Miltenyi Biotec; Bologna, Italy), as described in the supplier protocol. CD56+ cells were re-plated on gelatin-coated dishes, expanded and used for further experiments within for 2-8 passages. When needed differentiation into myotubes was achieved by exposing confluent cultures to a differentiation medium composed of 2% horse serum, 30 µg/ml insulin and Pen/Strep in DMEM for 7 days.

Single nucleotide polymorphism (SNP) genotyping:

rs28357094 genotyping was performed using amplification refractory mutation system polymerase chain reaction method (ARMS-PCR) and then confirmed by Applied Biosystems TaqMan SNP

genotyping assays and end-point allelic discrimination on an ABI-7000 SDS instrument. Primers and PCR conditions are available upon request.

Deflazacort (DFZ) treatment:

$7x10^4$ cells per well were plated on gelatin-coated 35 mm wells. Proliferating myoblasts or differentiating myotubes were treated with vehicle (untreated cells), 0.1 µM or 1 µM DFZ for 3 days, then cells were lysed for western blotting or RNA extraction.

Real Time PCR analysis:

Total RNA was isolated from myoblasts and myotubes using Trizol (Life Technologies; Carlsbad, CA, USA). For all conditions, 1µg of total RNA was reverse-transcribed to cDNA according to the protocol of SuperScript III Reverse Transcriptase (Life Technologies; Carlsbad, CA, USA). Transcript levels were measured using SYBR Green Real-Time PCR (Applied Biosystem; Foster City, CA, USA) using the ABI PRISM 7000 sequence detection system. The *TBP* (TATA box binding protein) gene was used as internal control (primers sequences are available upon request). Relative expression (R) was then calculated with the ΔCT method (Piva et al. 2012).

Western Blot:

Proliferating myoblasts and differentiated myotubes were washed twice in PBS, and incubated in 100 µl of 10 mM Tris (pH 6.8), 1 mM EDTA, proteinase inhibitor cocktail (Roche; Basel, Switzerland) for 15 minutes on ice. 10 µl of 10% SDS were added to each well, cells were mechanically broken with a cell scraper, and the protein lysate was vortexed and passed through a pipette tip. Protein concentration was determined with BCA Protein Assay Kit (Thermo Scientific-Pierce; Waltham, Massachusetts, USA) and 20 µg of protein per sample were loaded onto 10% or 12% glycine-tris large-sized acrylamide gels. Proteins were blotted onto a 0.45 µm nitrocellulose membrane (GE Healthcare; Waukesha, WI, USA) in transfer buffer. Membranes were saturated in 5% TBST milk at room temperature for 1 h and incubated with goat polyclonal anti-OPN antibody (O3389, 1:500; Sigma-Aldrich, St. Louis, MO, USA) or mouse monoclonal anti-actin antibody (MAB1501, 1:5.000; Millipore; Billerica, MA, USA) in 5% TBST milk at 4°C overnight. Appropriate secondary HRP-conjugated antibodies were used and bands were visualized by enhanced chemiluminescence (Millipore; Billerica, MA, USA). Integrated optical density of each band was calculated with QuantityOne commercial software (Bio-Rad; Hercules, California, USA) and normalized to actin. For validation purposes, protein lysates from select (two DMD and two control) cultures were blotted with two alternative anti-OPN antibodies: the ab8448 Abcam (Cambridge, UK) rabbit polyclonal anti-OPN antibody raised against the CKSKKFRRPDIQYPD peptide

(aa 170-183 of human OPN), and the AKm2A1 (sc-21742) Santa Cruz Biotechnology (Dallas, TX, USA) mouse monoclonal antibody against recombinant murine OPN.

Osteopontin (OPN) silencing:

Differentiating myotubes were transiently transfected with siRNA sequences targeting all known alternatively spliced OPN isoforms (NM_000582: SASI_Hs01_00174866 and SASI_Hs01_00174867, Sigma; St. Louis, MO, USA) or scramble control siRNA with Lipofectamine 2000 reagent. 50 nM of siRNA oligos and 15 µl of Lipofectamine in 1 ml Optimem (Invitrogen; Carlsbad, CA, USA) were used for 35 mm well. Myotubes were lysed on ice at 7 days of differentiation.

Statistical analyses:

Osteopontin expression was evaluated as four distinct outcomes: SPP1 mRNA evaluated by RT-PCR (normalized to TBP expression); OPN protein expression evaluated by densitometry of the 50 kDa Western blot band; OPN protein expression evaluated by densitometry of the 55 kDa Western blot band; and OPN protein evaluated by densitometry of the two bands combined. All Western blot intensities were normalized to ACTB band intensity. The following variables, pertaining to patients from whom original muscle biopsies were obtained, were used to group corresponding primary cell cultures: DMD patient vs. healthy control, and rs28357094 genotype (TT vs. TG). Due to the relative rarity of the homozygous GG genotype, it was not possible to obtain a sufficient number of GG cultures for a meaningful statistical comparison, so these were not included in this study. However, the TT to TG comparison may be considered adequate in the light of the dominant effect of the rs28357094 SNP described in DMD (Pegoraro et al. 2011). Outcome differences between groups were compared with the Mann-Whitney U test. Outcome changes within groups after myotube differentiation were evaluated by paired Wilcoxon test, while outcome changes within groups with increasing concentrations of DFZ were evaluated by repeated measures ANOVA. Independent and concurrent effects of biopsy variables (dystrophin deficiency and rs28357094 genotype as categorical variables) and DFZ concentration (as a quantitative variable), as well as of their interactions, were evaluated in repeated measures ANCOVA models, distinctly for myoblast and differentiated myotube cultures. Statistical significance was set at $p < 0.05$.

Results

Human myoblasts, myotubes and skeletal muscles express OPN isoforms of different molecular weight

We compared OPN expression patterns in DMD proliferating cells, differentiated myotubes and muscle biopsies by western blot. As shown in Figure 1 (panel A), OPN was detected as a single 55 kDa band in DMD myoblasts, as two distinct bands of approximately 55 and 50 kDa in differentiated DMD myotubes, and as a single 50 kDa band in muscle biopsies from DMD boys. The same expression pattern was observed in control myoblasts, myotubes, and muscles, while recombinant human OPN was observed at a molecular weight (MW) between 55 and 50 kDa (see Supplementary Material). We checked the specificity of both observed OPN bands by performing a transient siRNA-mediated OPN silencing in differentiated myotubes, in which both 55 and 50 kDa bands were observed. After six days of differentiation, cultures were transiently transfected with siRNA oligonucleotides and collected 24h post transfection. Both 55 and 50 kDa bands were down-regulated to the same extent by two distinct siRNA sequences (Figure 1; panel B-D), confirming that the observed bands were indeed the products of *SPP1* gene translation. Since the presence of the two bands appeared to vary according to muscle maturation, throughout this work we quantified OPN protein both as individual bands and as the sum of both bands. Validation experiments with two alternative anti-OPN antibodies confirmed an OPN band at 55 kDa (Supplementary Figure 1).

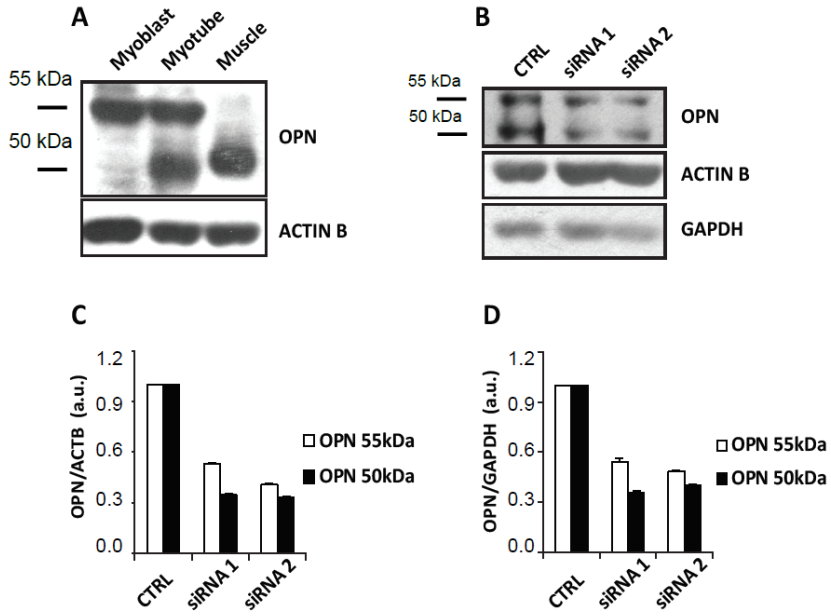


Figure 1. OPN protein is present with different molecular weight in human myo- blasts, myotubes and muscle. (A) Western blot of total protein lysates obtained from DMD myoblasts, myotubes and muscles showing a developmentally regulated OPN isoform expression. (B) Western blot of total protein lysates obtained from control myotubes transfected with mock and two distinct siRNA oligos against human OPN. Down-regulation of both 55 and 50 kDa bands was quanti- fied with densitometric image analysis (C, D).

Differentiation into myotubes increases both *SPP1* transcript and 50 kDa OPN expression, more markedly in DMD primary cultures

In order to define if OPN is upregulated in DMD primary muscle cells as it is in DMD muscle biopsy samples (Piva et al. 2012), total *SPP1* mRNA and OPN protein were isolated from eleven DMD myogenic cell lines and nine age-matched normal controls. Total *SPP1* mRNA level, evaluated by RT-PCR, and OPN protein expression, evaluated by Western blot, were quantified in myoblasts (Figure 2, panels A-D) and differentiated myotubes (Figure 2, panels E-H). In myoblasts, no differences were observed in OPN expression between DMD and control cultures, neither at the transcript nor at the protein level. After differentiation, there was an increase of *SPP1* mRNA in DMD cultures, although this was not statistically significant (paired Wilcoxon test = 0.053, see Figure 2, panel A compared to E), and, as expected from the initial analyses described above, a significant increase in OPN expression evaluated by the 50 kDa band in both DMD and control cultures (p = 0.021 and 0.015 respectively, see Figure 2, panel B compared to F). This increase was more marked in DMD cultures, although the Mann-Whitney test comparing the DMD vs. control myotubes was not significant. On the other hand, there were no significant changes in the expression of OPN evaluated as the 55 kDa band or both bands combined. Taken together, these results confirm that there is an increase in 50 kDa OPN expression after myotube differentiation, which might depend on increased *SPP1* transcription, and be more pronounced in dystrophin deficient myogenic cells. However, in isolated myogenic cell cultures we did not observe the dramatic upregulation of *SPP1* transcript and OPN protein, that has been previously described in dystrophin-deficient muscle tissue (3, 17).

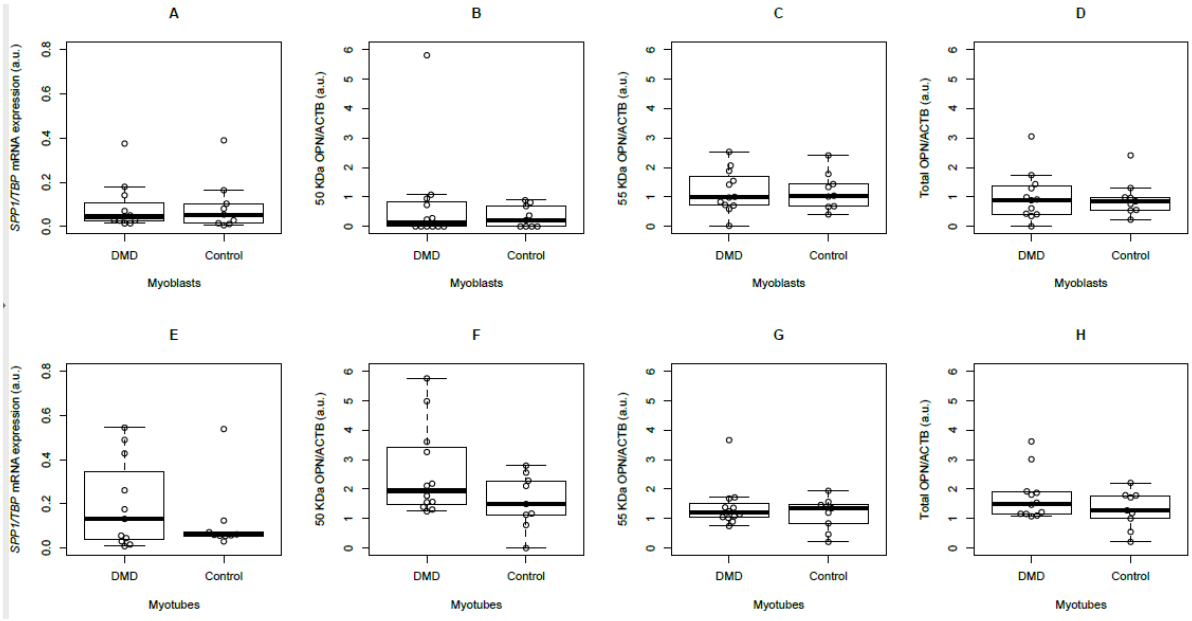


Figure 2. *SPP1* mRNA and OPN protein expression in DMD and control (WT) myoblast and myotube cultures. (A–D) Summarize data from myoblast cultures, while (E–H) illustrate data from myotube cultures. (A) and (E) refer to *SPP1* transcript expression levels assessed by RT-PCR; (B) and (F) refer to OPN protein expression evaluated by Western Blot as the 50 kDa band; (C) and (G) refer to OPN protein expression evaluated by Western Blot as the 55 kDa band; (D) and (H) refer to OPN protein expression evaluated by Western Blot as the two bands combined.

OPN protein levels are influenced by rs28357094 genotype in differentiated DMD myotubes

To verify if genotype at the rs28357094 SNP in the *SPP1* promoter predicts *SPP1* mRNA and/or OPN levels, 11 DMD (7 TT, and 4 TG) and 9 age-matched normal myogenic cell lines (5 TT, and 4 TG) were stratified based on their genotype. In myoblasts, there were no significant differences between genotypes neither in *SPP1* mRNA expression (Figure 3, panel A) nor in OPN protein expression evaluated as the 50 kDa band (Figure 3, panel B). However, we observed a significantly higher 55 kDa and total OPN protein expression in rs28357094 "TT" myoblast cultures, compared to "TG" (Mann-Whitney U test p = 0.018 for 55 kDa, and p = 0.003 for total, Figure 3, panels C and D). When considering DMD cultures only, this difference remained statistically significant for total OPN (p = 0.018), but not for 55 kDa OPN (p = 0.07). Interestingly, the situation seemed to be inverted in myotubes, where the TT genotype, compared to TG, was associated to significantly reduced 55 kDa and total OPN expression (p = 0.0043 and 0.018 respectively, Figure 3, panel G and H). The genotype difference in 55 kDa OPN expression remained significant, when considering DMD cultures only (p = 0.01). In myotubes, there were no other significant differences in *SPP1* mRNA or 50 kDa OPN expression between genotypes (Figure 3, panel E and F).

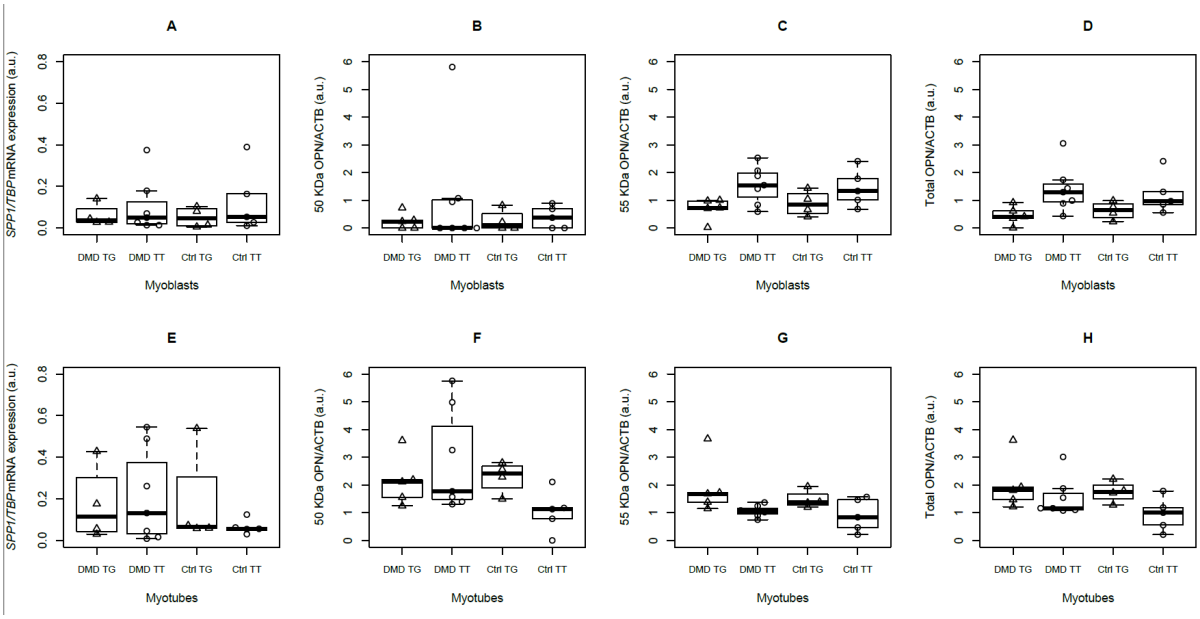


Figure 3. *SPP1* mRNA and OPN protein expression in DMD and control myoblast and myotube cultures, stratified by *SPP1* rs28357094 genotype. (A–D) summarize data from myoblast cultures, while (E–H) illustrate data from myotubes. (A) and (E) refer to *SPP1* transcript expression levels assessed by RT-PCR, (B) and (F) refer to OPN pro- tein expression evaluated by Western Blot as the 50 kDa band, (C) and (G) refer to OPN protein expression evaluated by Western Blot as the 55 kDa band, and (D) and (H) refer to OPN protein expression evaluated by Western Blot as the two bands combined.

Dystrophin deficiency and rs28357094 TG genotype may increase glucocorticoid responsiveness of the *SPP1* promoter

To test if DFZ treatment modulates *SPP1* mRNA or OPN protein in DMD myoblasts and myotubes, and if this response is modified by *SPP1* rs28357094 genotype, RT-PCR analysis and western blotting analyses were performed in DMD and control cell lines treated with 0.1 µM and 1 µM DFZ; results were then stratified by *SPP1* genotype. In myoblasts, DFZ treatment did not modify *SPP1* mRNA expression in control cultures. The same was true for DMD cultures from patients carrying the TT genotype, while a non-significant increase in transcript quantity was noted with in DFZ-treated DMD cultures from patients carrying the TG genotype (repeated measures ANOVA $p = 0.058$, Figure 4, panel A). In myotubes, baseline levels of *SPP1* mRNA expression were slightly higher, especially in DMD and rs28357094 TG samples, as stated above. When DFZ was added to the culture, there appeared to be a slight, non-significant decrease in control cultures, while DMD cultures showed slightly increased transcription levels, especially for TG samples, albeit with high variability and no statistically significant correlations (Figure 4, panel E).

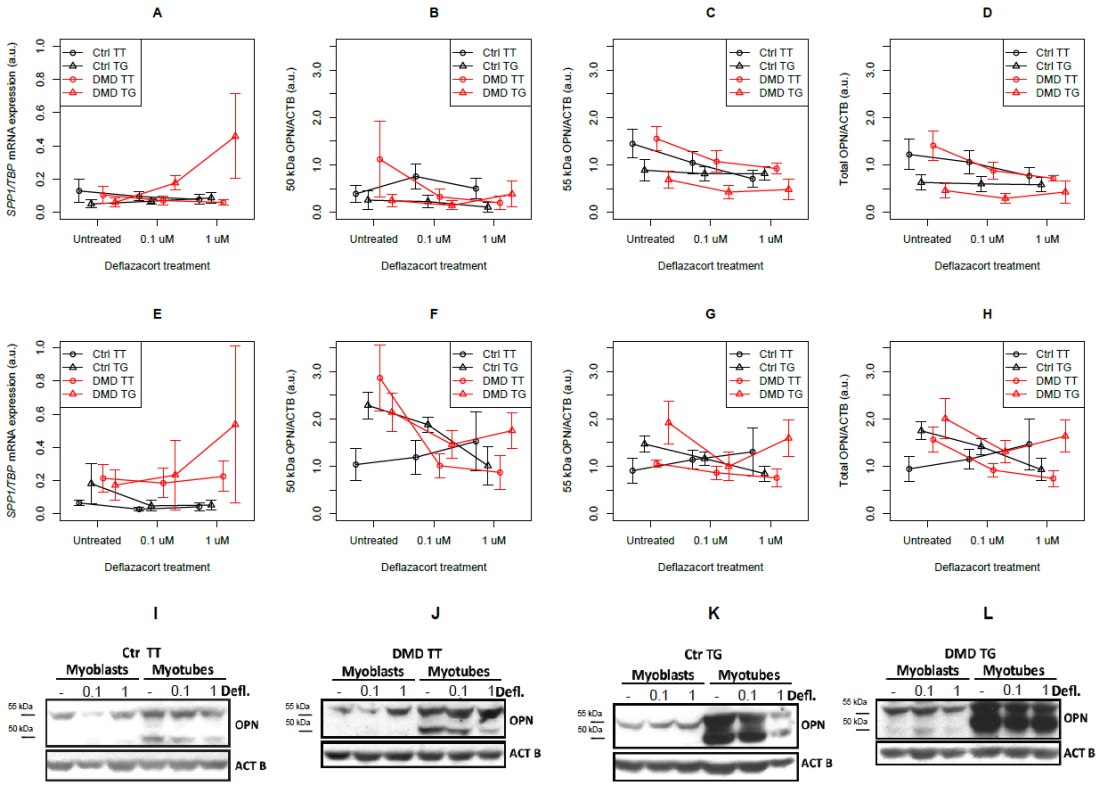


Figure 4. OPN protein expression after treatment with increasing concentrations of Deflazacort (DFZ). *SPP1* mRNA and OPN protein expression are represented as means (symbols) and their standard errors (bars). Lines connect data points for cultures from the same groups, at increasing DFZ concentrations. (A–D) refer to myo- blasts culture, while (E–H) refer to differentiated myotubes culture. (A) and (E) refer to mRNA expression; (B) and (F) to the 50 kDa OPN band; (C) and (G) refer to the 55 kDa OPN band; and (D) and (H) refer to the two bands combined. (I–L) show representative Western blot results from myoblasts and myotubes culture from each cell type and genotype group (control TT, control TG, DMD TT, and DMD TG).

		Outcomes in myoblast cultures				Outcomes in myotube cultures			
		SPP1 mRNA	50 kDa OPN	55 kDa OPN	Total OPN	SPP1 mRNA	50 kDa OPN	55 kDa OPN	Total OPN
Factors	Dystrophin deficiency	n.s.	n.s.	n.s.	n.s.	**0.029**	n.s.	n.s.	n.s.
	[DFZ]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	rs28357094 genotype	n.s.	n.s.	**0.001**	**0.0005**	n.s.	n.s.	0.050	**0.048**
Interactions	Dystrophin def. * [DFZ]	0.085	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Dystrophin def. * rs28357094	**0.029**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	[DFZ] * rs28357094	**0.020**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Dystrophin def. * [DFZ] * rs28357094	0.073	n.s.	n.s.	n.s.	n.s.	**0.033**	n.s.	**0.041**

SPP1: osteopontin (Secreted PhosphoProtein) gene. OPN: osteopontin. [DFZ]: deflazacort concentration (μM). n.s.: not significant.

Table1: Analysis of covariance (ANCOVA) of SPP1 mRNA and OPN protein expression: p-values for factors (dystrophin deficiency, rs28357094 genotype, and DFZ cpncentrations) and their interactions.

Effect of deflazacort treatment and OPN protein expression as a function of rs28357094 genotype

We then analyzed changes in OPN protein expression in myogenic cell cultures treated with DFZ. In myoblasts, the 50 kDa OPN band was detected at low levels at baseline, except in DMD cultures carrying the TT genotype which showed higher levels. In this subgroup, the 50 kDa band intensity decreased slightly, although not significantly, with increasing concentration of DFZ (Figure 4, panel B). Conversely, DMD "TG" cultures showed an increase of 50 kDa band intensity at 1 µM DFZ, which was significant in the repeated measures ANOVA model for increasing DFZ concentration ($p = 0.012$, Figure 4, panel B). The 55 kDa band of OPN and total OPN showed a slight decrease with increasing DFZ concentrations in all subgroups, which was statistically significant only in control samples carrying the TT genotype for both 55 kDa and total OPN (repeated measures ANOVA $p = 0.038$ and 0.033 respectively, Figure 4, panels C and D). In myotubes, expression of the low molecular weight band was higher as stated above, but we did not observe any significant effects of increasing DFZ concentrations on OPN protein quantity, assessed by either OPN band or as a whole, in any of the subgroups, although DMD "TG" cultures did show the highest average intensity of the both 50 and 55 kDa OPN band with 1 µM DFZ (Figure 4, panels F to H). With 1 µM DFZ treatment, total OPN was higher in DMD myotubes carrying the TG, rather than TT genotype ($p = 0.047$). Representative Western blots for the different groups are shown in Figure 4, panels I to L, and all blots used for quantitation are available as Supplementary Material.

Multivariate analysis confirms the concurrent effect of dystrophin deficiency and rs28357094 genotype in increasing glucocorticoid responsiveness of the *SPP1* promoter

Taken together, the results presented above suggest that osteopontin expression in myogenic cells, at the transcript and protein level, is influenced by the complex interaction of several factors: muscle cell maturation, dystrophin deficiency, rs28357094 genotype, and glucocorticoid (DFZ) treatment. In order to dissect independent and concurrent effects of these variables on the outcomes of interest (*SPP1* transcript and OPN protein bands), we devised a repeated measures ANCOVA model evaluating the correlation of outcomes with different DFZ concentrations, with covariates for dystrophin deficiency and rs28357094 genotype, and interaction terms between covariates, in both myoblast and myotube cultures. The ANCOVA analysis of *SPP1* mRNA expression in myoblasts showed significant interaction with rs28357094 genotype for both dystrophin deficiency and DFZ concentration ($p = 0.029$ and 0.020 respectively). This interaction is driven by the DFZ-induced, dose-dependent mRNA increase in DMD "TG" samples (Figure 4, panel A). Again in myoblasts, the ANCOVA analysis of OPN protein band intensity showed strong significant independent effects of rs28357094 genotype on 55 kDa and total OPN ($p = 0.001$ and 0.0005 respectively), corresponding to the increased 55 kDa and

total OPN band intensity in Western blots from "TT" cultures at baseline (Figure 3, panels C and D). The very low intensity of the 55 kDa OPN band in myoblasts accounts for similar findings in 55 kDa and total OPN. In myotubes, dystrophin deficiency had a significant, independent effect in increasing *SPP1* transcript ($p = 0.029$, see Figure 2, panel E), while the independent effects of rs28357094 on 55 kDa and total OPN protein quantity were barely significant ($p = 0.050$ and 0.048 respectively), although these effects were in the opposite direction as observed in myotubes (see Figure 3, panels G and H, as opposed to panels C and D). Furthermore, the complex interaction terms of all three covariates (dystrophin deficiency, rs28357094 genotype, and DFZ concentration), was significant in the ANCOVA analysis of 50 kDa and total OPN in myotubes ($p = 0.033$ and 0.041 respectively).

Discussion

The role of genetic modifiers of DMD is becoming increasingly interesting given the potentially relevant implications they have for selection of homogeneous groups of patients, deeper knowledge of the pathophysiology of dystrophin deficiency, and planning of novel therapeutic strategies. The availability of genotyping chips and next generation sequencing (NGS) techniques has allowed genome-wide study of genetic variations in large populations in a cost-effective and highly efficient way (Bello et al. 2016). However, once novel associations are established between SNPs and disease phenotypes, we are faced with the problem of linking genetic variation to protein function in the context of cellular molecular mechanisms (Cayer et al. 2016). Osteopontin as a DMD modifier epitomizes this challenge. The discovery that a single nucleotide polymorphism (SNP) in the promoter region of the *SPP1* gene is able to modify muscle strength in DMD (Pegoraro et al. 2011) has represented the proof-of-principle that genetic modifiers have a role in DMD disease progression, but it also raised several questions that still await for a definitive answer: is osteopontin secretion damaging or beneficial in the dystrophic muscle microenvironment? Is the transcriptional effect of the polymorphism the actual disease-modifying mechanism? And is *SPP1* genotype a modifier of disease progression, or of response to treatments?

In this study we started to dissect the complex molecular mechanisms underlying the effect of *SPP1* genotype on ostepontin protein expression, and the effect of steroid treatment in different *SPP1* genetic backgrounds, to explain some of the observed variability in DMD disease progression.

First of all, our results suggest that OPN expression may be developmentally regulated in muscle. In particular, OPN was identified in proliferating myoblasts as a 55 kDa band, in differentiated myotubes as both a 55 and a 50 kDa band, and in mature muscle as a single 50 kDa band. Gene silencing

experiments, targeted to *SPP1*, confirmed that both observed bands correspond to osteopontin isoforms. A certain degree of variability in the ratio between the two bands was observed in the experimental setting. We suppose that this variability may be in part related to the various percentage of non-myogenic cells present in each cell line (Partridge et al. 1989) and in part to the degree of myoblasts differentiation to myotubes (Cheng et al. 2014). Even if, cell cultures were enriched in CD56 positive cells (Belles-Isles et al. 1993) in order to minimize these confounding factors, some contaminating fibroblasts may have escape selection and be responsible of the observed variability. It is hard to predict if the observed different molecular weight OPN bands arise from *SPP1* splicing isoforms, from different post-translational modifications, or a combination of the two. It is well known that the *SPP1* transcript is subject to alternative splicing generating different isoforms that show specific expression and roles in different cell contexts (Gimba e Tilli 2013; Saitoh et al. 1995), and this also applies to skeletal muscle (Many et al. 2016a). The molecular weights reported for splicing isoform OPN-a and OPN-b (54 kDa and 50 kDa, respectively) (Many et al. 2016a) are close to those of the observed bands, and data from our laboratory show a high-level expression of both OPN-a (full length) and OPN-b (Δ exon 5) transcripts in human myoblasts (see the section: "Aim 2: does the expression of different OPN splicing isoforms contribute to the effects of OPN as a DMD modifier?"). However, fully dissecting OPN isoform expression in muscle is beyond the purpose of this paper. Furthermore, OPN is subject to extensive post-translational modifications by glycosylation, phosphorylation, and sulphation, and possibly also by cross-linking and proteolytic cleavage (Pagel et al. 2014).

To add to the hypothesis of a regulation of osteopontin during development, in DMD cultures we observed an increase of *SPP1* mRNA and of the 50 kDa OPN isoform when shifting from myoblasts to myotubes. These modifications were observed also, albeit to a lesser extent, in control muscle cultures. However, the differential expression in transcription and translation of osteopontin between normal and DMD cultured cells did not match the definite overexpression of ostepontin observed in DMD muscle tissue compared to controls (Piva et al. 2012; Haslett et al. 2002; Zanotti et al. 2011). The developmental shift in OPN molecular weight, regardless of its actual molecular basis, might reflect the shift between different biological roles of OPN, first as a chemotactic cytokine in the acute phase of inflammation, and then as a regeneration-enhancing matricellular protein (Uaesoontrachoon et al. 2013; Pagel et al. 2014).

As a model system, we used DMD proliferating myoblasts and differentiated myotubes. While this model offers several advantages, such as the use of cells from patients with definite *SPP1* genotypes

and the possibility to manipulate the physico-chemical environmental, an important drawback is represented by the lack of stress-induced muscle damage, and its downstream consequences. DMD pathophysiology is the direct consequence of dystrophin deficiency in muscle fibers, resulting in muscle fiber fragility and contraction-mediated injury, and leading to asynchronous cycles of segmental necrosis and regeneration, which are at first effective but eventually fail and give way to fibrosis (Petrof et al. 1993; Chen et al. 2005). Contraction-induced sarcolemma rupture triggers a variety of molecular changes including release of mitogenic factors (G. Chen et al. 1994; R. E. Allen et al. 1995; Tatsumi et al. 1998), cytokines (Bakay et al. 2002), and reactive inflammatory signature molecules (Uaesoontrachoon et al. 2008; Porter et al. 2002) that modify the muscle microenvironment. Since osteopontin is induced by skeletal muscle injury (Hoffman et al. 2013b; Uaesoontrachoon et al. 2013; Barbosa-Souza et al. 2011; Hirata et al. 2003; Kuraoka et al. 2016), and muscle cells remain intact in cell culture, we feel that the reduced overexpression of OPN in myoblasts and myotubes is probably due to the lack of the cascade of events triggered by the muscle damage itself. Moreover, since osteopontin is also secreted from infiltrating macrophages in the foci of muscle cell degeneration (Vetrone et al. 2009; Uaesoontrachoon et al. 2008), the lack of inflammatory cells in our model may be an important factor in explaining the lack of OPN overexpression we found in cultured DMD cells compared to *in vivo* findings.

Another intriguing issue is the role of the rs28357094 single nucleotide polymorphism in gene transcription/translation, and how these events are in turn capable to modulate DMD phenotype expression. *In vitro SPP1* transcription studies (Giacopelli et al. 2004; Barfield et al. 2014) predict that DMD patients carrying the more common TT genotype at rs28357094, who lose ambulation later and show a greater grip strength compared to TG/GG patients (Pegoraro et al. 2011; Bello et al. 2015), should have higher *SPP1* promoter activation, and hence higher osteopontin levels in muscle. However, rs28357094 genotype did not correlate with either the level of *SPP1* mRNA or the amount of OPN protein in DMD muscle biopsies, taken at diagnosis prior to any treatment (Piva et al. 2012).

In DMD proliferating myoblasts, we did not observe any difference between genotypes in *SPP1* mRNA expression, but we found a significant overexpression of the 55 kDa OPN band in cells carrying the TT genotype compared to TG. This result is in line with *in vitro* transcriptional activity studies of the *SPP1* gene in different rs28357094 genotypes (Giacopelli et al. 2004; Barfield et al. 2014) and with the hypothesized developmental role of the OPN molecular weight transition, that predicts a greater expression of the higher molecular weight band in the early phases of myoblast proliferation. In differentiating myotubes, the downregulation of the 55 kDa band in the TT genotype fits with this

hypothesis. On the other hand, the expected upregulation of the 50 kDa band in TT DMD culture did not reach significance because of a very large standard deviation. However, the TT myotubes displayed the highest level of osteopontin compared to TG.

Glucocorticoids are beneficial in DMD (Matthews et al. 2016; Gloss et al. 2016), and are considered standard of care in the management of the disease. More importantly, they are the only medication available to all DMD patients regardless of their specific *DMD* gene mutation, and at present, the most diffusely prescribed treatment in DMD. It has been shown that the effect of rs28357094 in DMD is enhanced by glucocorticoids (Bello et al. 2015; van den Bergen et al. 2015a), and it has been proposed that osteopontin may be envisaged as a pharmacodynamic modifier of glucocorticoid response in DMD (Pegoraro et al. 2011; Bello et al. 2015). Among steroids, deflazacort (DFZ) is emerging as potentially more effective (Bello et al. 2015) and more tolerable than prednisone (Bonifati et al. 2000; Griggs et al. 2016), but with both these drugs response to treatment is variable, some patients showing greater clinical benefit than others. Molecular bases of this differential efficacy of steroids are largely unknown. In this study, we treated both proliferating myoblasts and differentiating myotubes, carrying different *SPP1* rs28357094 genotypes, with two DFZ regimens (low and high dosage), to verify if osteopontin has a role in steroid response in DMD. In both myoblasts and myotubes, *SPP1* mRNA levels were higher in the TG than TT genotype, although with high variability in the data. A multivariate analysis, taking in account the concurrent effect of dystrophin deficiency and rs28357094 genotype, showed a significant interaction. These results are in line with the hypothesis that glucocorticoid receptor elements (GREs) are active in the promoter region of the *SPP1* gene (Barfield et al. 2014). The rs28357094 polymorphism is located in the *SPP1* promoter region, 66 bp upstream of the transcription start, and it has been shown to modify the binding affinity for the SP1 transcription factor (Giacopelli et al. 2004). In DFZ-treated cells with a TT genetic background the *SPP1* gene transcription is driven by SP1, whereas in TG cells, where the G polymorphism interferes with the binding of SP1 (Giacopelli et al. 2004), the GRE elements, activated by DFZ, may promote a very efficient and sustained gene transcription. The role of enhancer elements in the *SPP1* promoter has been already hypothesized to explain the rs28357094 female-specific genotype effect in modifying muscle size in female adult volunteers (Eric P. Hoffman et al. 2013b; Barfield et al. 2014). Allele-specific reporter assays *in vitro* showed that the G allele responds to estrogen treatment with a 3-fold increase in luciferase activity compared to untreated cells, likely due to the interaction between estrogen enhancer elements and the more proximal SP1 transcription factor site (Barfield et al. 2014). A limit of our experimental approach, that could potentially affect sensitivity, is the dominant genotype

model. Due to the scarce availability of GG genotype muscle cell, we compared homozygous TT cells with heterozygous TG. While the modifier function of the SNP was observed in DMD populations as a dominant effect, at the molecular level homozygous GG cells might present a clearer SNP effect.

When switching from transcript to OPN protein level analyses, DFZ effects become more intricate. In TG genotype myoblasts, the 55 kDa OPN band showed no changes with DFZ treatment, while the 50 kDa OPN band, expressed at low level in myoblasts, resulted significantly increased with treatment. This is in line with the model of a G-allele specific upregulation of OPN by glucocorticoids. Conversely, in TT myoblasts, the 55 kDa OPN band showed a significant decrease in DFZ-treated cells. Trajectories in OPN protein expression, after DFZ treatment, did not show significant modifications in myotubes, although those with the TG genotype showed the highest average intensity of the 50 kDa OPN band. The complex interaction among dystrophin deficiency, rs28357094 genotype and DFZ concentration was significant in multivariate analysis for myotubes. Thus, it seems that DFZ treatment lead to a significant decrease of OPN in TT myoblasts and myotubes, whereas TG cells showed subtle changes. The finding that *SPP1* mRNA expression does not exactly predict OPN protein expression levels may be related to post-transcriptional and post-translational regulatory steps (Pagel et al. 2014), or to limits of our study design, that did not measure secreted OPN in the culture medium, leaving the possibility that DFZ treatment may affect the amount of the soluble protein. Taken together, our data suggest that OPN transcription and protein synthesis are influenced by DFZ treatment and that the effect of DFZ is fine-tuned by rs28357094 genotype and dystrophin absence in primary human muscle cells.

Given the results of this study, our unifying model to explain the effect of *SPP1* rs28357094 in modulating phenotype expression in DMD postulates as follows. Lack of dystrophin in skeletal muscle fibers induces osteopontin expression both by muscle cells and by infiltrating inflammatory cells (Pagel et al. 2014). While osteopontin is beneficial to DMD muscle in the early phases of the regenerative events that follows muscle injury (Uaesoontrachoon et al. 2013), its chronic overexpression may hinder regeneration (Paliwal et al. 2012). Indeed, osteopontin expression in skeletal muscle is strictly regulated in time: it is induced approximately 100-fold within 1 day after muscle injury, it remains expressed at high level during regeneration, and falls back to baseline by 16 days, when regeneration is complete (Eric P. Hoffman et al. 2013b). The upregulation of *SPP1* transcript and OPN observed in our experiments recapitulate these phenomena in the myogenic cell cultures. In a *SPP1* rs28357094 TG genetic background, glucocorticoids, through activation of GRE elements, interfere with the physiological SP1-mediated activation of the promoter, and result in chronic ostepontin

overexpression. In this scenario, disregulated *SPP1* expression may add to the desynchronization of damage-related gene expression patterns in dystrophic muscle, which has been shown to be a driver of failed regeneration and fibrosis (Dadgar et al. 2014).

Further research is needed to better understand the role of specific OPN isoforms, the various post-translational modifications of the protein, and the effects of steroids in the various *SPP1* genetic backgrounds, which might also be relevant to inflammatory diseases beyond DMD.

Acknowledgements

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Aim 2: does the expression of different OPN splicing isoforms contribute to the effects of OPN as a DMD modifier?

Introduction

Osteopontin (OPN) is a member of the small integrin-binding N-linked glycoprotein family, coded by the *SPP1* gene (Denhardt et al. 2001). OPN is expressed in a wide range of cells, including epithelial cells, osteoblasts, osteoclasts, macrophages, lymphocyte and muscle cells.

There are 3 main isoforms (a, b and c) of OPN due to alternative splicing. While OPNa is the full length isoform coded by 7 exons (~54kDa), OPNb lacks exon 5 (~50kDa) and OPNc lacks exon 4 (~47kDa). Recently, two additional “rare" isoforms have been described (4 and 5) (Lin et al. 2015). The exons which are spliced out in OPNb and OPNc are important sites for post-translational modifications of the protein. Exon 5 is a site of serine and threonine phosphorylation, while exon 4 is a site for transglutaminase cross linking (Anborgh et al. 2011), which is thought to modify protein function by altering the conformational state of OPN and thereby increasing its cell binding ability (Kaartinen et al. 1999; Li et al. 2015; Christensen et al. 2016). Therefore, OPNc may be unable to form polymeric structures and have reduced collagen binding capacity. All the isoforms share several functional sites, including the RGD domain, necessary for the pro-inflammatory action of the protein (Many et al. 2016b), the SVVYGLR sequence, a CD44 binding domain, and cleavage sites for thrombin and matrix metalloproteases (MMPs).

OPN is one of the most studied protein in the oncologic field (Gimba and Tilli 2013; Blasberg et al. 2010; Tilli et al. 2012; Nakamura et al. 2015; Ferreira et al. 2016; Shi et al. 2014; X. Sun et al. 2015). More recently it has emerged as a relevant actor in the skeletal muscle disease scenario. In particular, it has been shown to exert an active role during muscle damage and regeneration, promoting infiltration of inflammatory cells and myotube differentiation (Pagel et al. 2014; Uaesoontrachoon et al. 2013, 2008).

Furthermore, osteopontin is strongly overexpressed in dystrophic muscle tissue, and a SNP in the *SPP1* promoter region (rs28357094) has been shown to modulate the severity of DMD (Pegoraro et al. 2011; Piva et al. 2012;). rs28357094 is able to modulate *SPP1* transcription because interferes with a SP1 binding site located 66 bp upstream in the *SPP1* promoter region (Giacopelli et al. 2004). DMD patients carrying the G allele (GG or TG) decline more rapidly compared with patients carrying the TT genotype (Pegoraro et al. 2011; Bello et al. 2012, 2015).

While in human patients the rs28357094 G allele, expected to reduce *SPP1* expression, was associated with a more severe DMD phenotype, in the murine model of DMD, the *mdx* mouse, genetic ablation of OPN in a double mutant model lead to an increase in muscular strength, a decrease of fibrosis, and a shift to pro-regenerative macrophage phenotype (Vetrone et al. 2009; Capote et al. 2016). This discrepancy between human and animal model data has been partly explained with the hypothesis that the rs28357094 might modulate response to glucocorticoid corticosteroid (GC) treatment rather than modulating disease progression in itself, with supporting evidence in observational studies of DMD cohorts genotyped for the *SPP1* SNP and characterized for GC treatment (Bello et al. 2015), and *in vitro* studies of DMD myogenic cells treated with GCs (Vianello et al. 2017).

The existence of several OPN splicing isoforms, which have been shown to exert specific functions in cancer diffusion, adds one more layer of complexity to the mechanisms by which OPN modulates dystrophic muscle pathology. OPNa, OPNb, and OPNc have been shown to be expressed in human dystrophic muscle, OPNa being the most abundantly expressed and the most pro-inflammatory (Many et al. 2016b). Based on this evidence and on the lack of information about OPN isoforms in myopathies, we have decided to study the level of expression of the main splicing isoforms of OPNs (a, b and c) in muscle biopsies and myotubes from DMD and control patients. Moreover, we used as pathological control, polymyositis (PM) muscle biopsies. In comparison with DMD muscles, where the inflammatory reactions are moderate, PM muscles show a dramatic inflammation and it has been hypothesized that OPN may have a role in the inflammatory process itself (Xiao et al. 2015). In order to discriminate between dystrophin-related OPN isoforms expression regulation and inflammatory-related phenomenon we studied the pattern of expression of OPNa, OPNb and OPNc in DMD and PM muscles. .

Materials and Methods

Human myotubes

DMD and control myoblasts were provided by the Telethon tissue bank and obtained as previously described (Vianello et al. 2017).

Patients biopsies

Table1: Data of patients and controls.

				rs28357094 SNP genotype	
	number of subjects	sex	mean age at biopsy (year)	TG	TT
Controls	9	9 males	4.13	4	5
DMD	10	10 males	5.3	5	5
Polymyositis (PM)	11	4 males and 7 females	52.4	4	7

All skeletal muscle biopsies were selected from the biobank of the Neuromuscular Center of the University of Padua. The diagnosis of DMD was based on absence of dystrophin in skeletal muscle by Western Blot, and/or out-of-frame or nonsense *DMD* mutations. Biopsies had been taken prior to any pharmacological treatment.

Single nucleotide polymorphism (SNP) genotyping

rs28357094 genotyping was performed using amplification refractory mutation system polymerase chain reaction (ARMS-PCR), and confirmed by Applied Biosystems TaqMan SNP genotyping assays with end-point allelic discrimination on an ABI-7000 SDS instrument. Primers and PCR conditions are available upon request.

Real Time PCR (RT-PCR)

Total RNA was isolated from myoblasts and patients' skeletal muscle biopsies using Trizol (Life Technologies; Carlsbad, CA, USA). For all conditions, 1.5 μg of total RNA was reverse-transcribed to cDNA according to the protocol of SuperScript III Reverse Transcriptase (Life Technologies; Carlsbad, CA, USA). Transcript levels were measured using SYBR Green Real-Time PCR (Applied Biosystem; Foster City, CA, USA) using the ABI PRISM 7000 sequence detection system. β2-Microglobulin and β-Actin genes were used as internal control (primer sequences are available upon request). Relative expression was then calculated with the ΔCT method.

Statistics

Data are expressed as mean ± SEM. Comparisons of expression values between groups were performed using Student's t-test.

Results

DMD muscle tissues, but not DMD myotubes, show increased expression of OPN isoforms

In order to define if the main OPN isoforms (OPNa, OPNb, and OPNc) are upregulated in DMD patients compared to healthy controls, total RNA was isolated from muscular biopsies, and *SPP1* expression was quantified by RT-PCR. As shown in Figure 1a, the three isoforms of OPN were strongly over-expressed (about 40-fold) in skeletal muscle extracts from DMD patients, compared to healthy controls (age: 2 to 9 years). In DMD muscle tissues, the OPNb isoform (Δ-exon 5) was more abundant (0.042 ± 0.013) than OPNa (full length, 0.018 ± 0.006) and OPNc (Δ-exon 4, 0.018 ± 0.006).

Myotubes from both DMD and healthy individuals express all OPN isoforms with no significant differences between DMD and control cultures (Figure 1b).

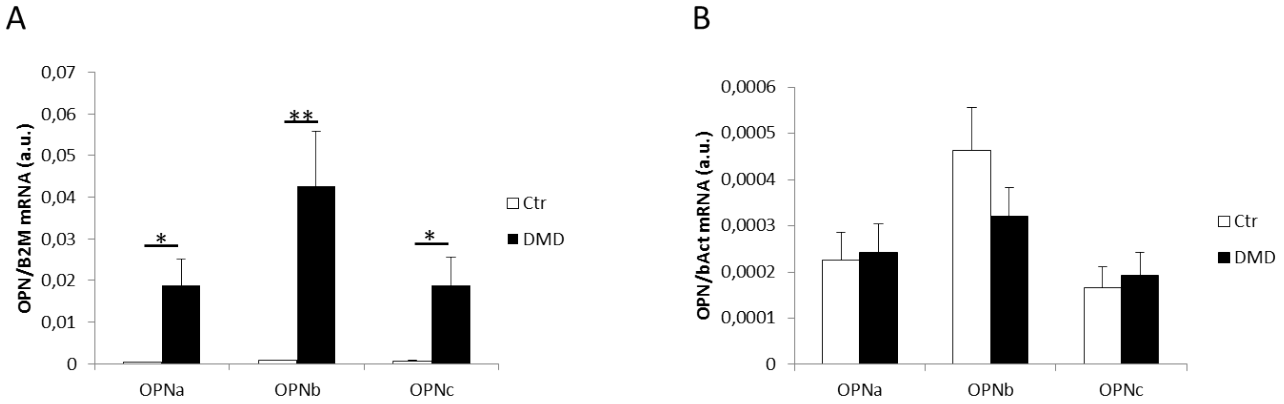

Figure 1. *SPP1* mRNA isoforms expression in DMD and control (Ctr) skeletal muscle and myotube cultures. Transcript expression of *SPP1* splicing isoforms, assessed by RT-PCR in (A) muscular biopsies, and (B) myotube cultures from DMD and healthy individuals. * $P < 0.05$.

Age influences OPN isoform expression in muscle biopsies, without modification of expression ratio between isoforms.

To assess if the relative levels of OPNa, OPNb, and OPNc transcripts vary with age, we stratified expression data in muscle biopsy from DMD individuals by age at the time of the biopsy. We observed a decrease of all OPN isoforms with increasing age in older boys, with lower values in boys who were 6 years old or older (Figure 2a). OPNb in any age range is the most upregulated isoform (Figure 2b). These results suggest that OPNa and OPNc play a role in the first phase of disease progression, when inflammation is still going on, while in a second phase, in which there is a progressive substitution of the muscular tissue with fibrotic tissue, OPNa and OPNc expression are down-regulated, but not

OPNb. In controls muscle the relative levels of OPN isoforms are not affected by the age of the biopsy.

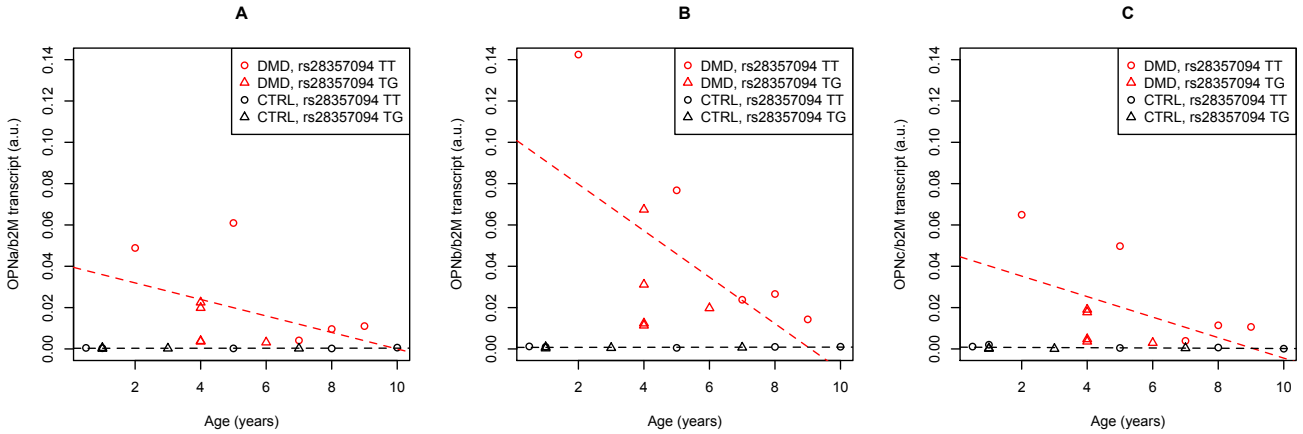

Figure 2. OPN isoforms expression level in DMD patients relative to age. Panels A, B and C refer respectively to OPNa, OPNb and OPNc expression level assessed by RT-PCR, according to the age of the patients at the time of the biopsies, stratified by diagnosis (DMD, healthy controls) and rs28357094 genotype (TT or TG).

rs28357094 genotype influence OPN isoform expression.

To verify whether the G allele at rs28357094 of the *SPP1* gene is associated with less OPN transcription (Giacopelli et al. 2004; Barfield et al. 2014), the DMD patients and controls were stratified based on their genotype. Similar to what observed by Piva et al. (Piva et al. 2012), TG patients tended to express less OPN isoform than TT patients (OPNa 0.01±0.004; 0.02±0.01; OPNb 0.02±0.01; 0.05±0.02; OPNc 0.009±0.003; 0.02±0.01), but this difference was not statistically significant (Figure 3).

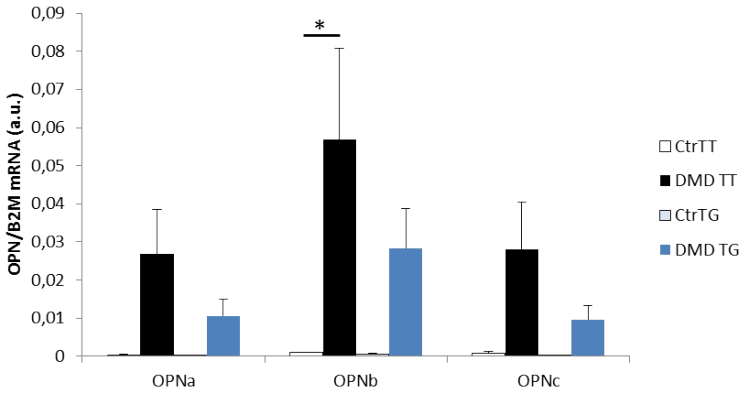

Figure 3. OPN isoforms expression stratified by *SPP1* rs28357094 genotype. OPN isoform expression level in DMD and control (Ctr) muscle biopsies assessed by RT-PCR stratified by rs28357094 genotype. Student's t-test with the level of significance set at 0.05.

Polymyositis (PM) patients express similar levels of OPNa, OPNb and OPNc.

In order to discriminate between dystrophin-related OPN isoforms expression regulation and inflammatory-related phenomenon we studied the pattern of expression of OPNa, OPNb and OPNc in PM muscles. PM patients express more OPN than controls, but without differences among the three splicing variants (Figure 4). The comparison between the level of expression of splicing variants between DMD and PM patients was not significant, however a trend towards a greater expression of OPNa (0.01±0.006 vs 0.036±0.01, respectively) and OPNc (0.01±0.006 vs 0.034±0.01, respectively) was observed in PM, and of OPNb in DMD (0.042±0.013 vs 0.031±0.011, respectively).

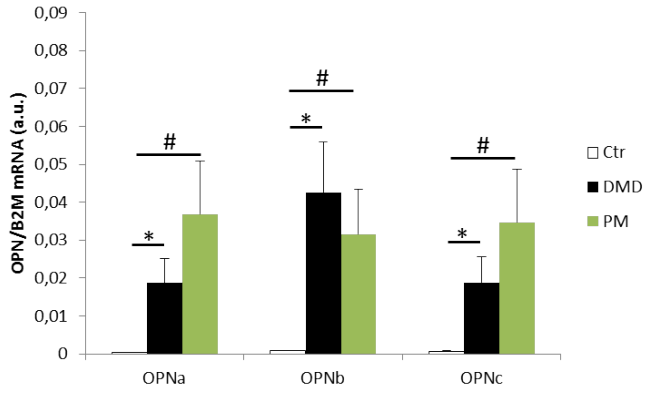

Figure 4. *SPP1* mRNA isoforms expression in control (Ctr), DMD and polymyositis (PM) skeletal muscle patients assessed by RT-PCR. Student's t-test with the level of significance set at 0.05, with $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$.

Discussion

We studied the three main isoforms of OPN (OPNa, OPNb and OPNc) in a relatively large collection of muscle biopsies from DMD, PM and age-matched healthy control individuals. Our data reveal that all the isoforms analyzed are more expressed in DMD muscles than controls, but not *in vitro* cultures where both DMD and controls myotubes express the same amount of OPN.

It is well know that OPN is overexpressed in *mdx* mice muscles in areas of cellular infiltration probably due to the regeneration process driven by myoblast activation in the focal lesions of mice dystrophic skeletal muscle (Uaesoontrachoon et al. 2008). In our DMD cohort, muscle biopsies were performed, for diagnostic purposes, at a mean age of 5 years when the inflammatory cascades, initiated by dystrophin deficiency, were predominating (Y.-W. Chen et al. 2005). Our findings support the role of OPN in the early stage of disease in DMD.

The observation that OPN isoforms were not overexpressed in DMD myotubes compared to controls has different explanations. *In vitro* myotubes cultures lack all pro-inflammatory factors that have a

pivotal role in inflammation. In addition, OPN implicated in muscle repair after injury is not exclusively produced by myogenic cells and cell culture lack all these other players. These results suggest that the role of OPN on *in vitro* differentiation of myoblasts (Pagel et al. 2014) is independent from the absence or presence of dystrophin. Moreover, the findings that OPN isoforms are more expressed in the first phase of the disease progression, during which inflammation is predominat and the muscle enacts a regeneration program, strongly confirm a role of OPN in muscle repair processes as shown also by OPN overexpression in PM muscles.

We did not find a role of rs28357094 genotype on OPN isoform expression pattern. At this point, we cannot completely exclude that the SNP located -66 bp from the promoter of the *SPP1* gene may modulate OPN isoforms expression but it is unlike since in our experiments the difference between genotypes was not significant. Furthermore, the study of OPNa, OPNb and OPNc in PM patients suggests a homogeneous expression pattern of the three isoforms in this inflammatory disease, as opposed to dystrophic muscle where OPNb is more expressed than OPNa and OPNc. There is a partial discrepancy between our findings and those of other laboratories which identified OPNa as the major isoform, followed by OPNb and OPNc (Many et al. 2016b). This could be due to technical variability (i.e. different primer designs in RT-PCR experiments), relatively small sample sizes leading to variable estimates of expression levels, or actual expression variability between individuals and groups due to unidentified factors. Anyhow, the fact that OPNc is the least expressed isoform seems established. Still, this isoform appears to have relevant biological roles as it is conserved along evolutions from dogs to humans. If the OPNc isoforms has reduced inflammatory potential, it might be most relevant in enhancing regenerative and reparatory processes (Pagel et al. 2014). Based on this evidence we can conclude that for a better comprehension of the role of OPN in dystrophic scenario it would be useful to study the active role of each OPN isoform, and their major source of production during inflammation.

Acknowledgments

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Aim 3: Collaborations

Are *LTBP4* (Latent Transforming growth factor β Binding Protein 4. rs2303729, rs1131620, rs1051303 and rs10880) and *CD40* (rs1883832) implicated in DMD pathology?

During my PhD I collaborated in the validation and characterization of two additional genetic modifiers of DMD: *LTBP4* and *CD40*.

Latent Transforming growth factor β Binding Proteins binds TGFβ in a latent complex within the extracellular matrix, preventing it from signaling to cells. The *LTBP4* locus was first associated with muscular dystrophy severity by genome-wide mapping in a genetically heterogeneous murine model: δ-sarcoglycan deficient mice carrying an intragenic *LTBP4* deletion presented an exacerbation of the phenotype (Heydemann et al. 2009). Subsequently, a human LTBP4 haplotype was associated with variable loss of ambulation (LoA) in 254 patients with severe dystrophinopathy (United Dystrophinopathy Project, UDP) (Flanigan et al. 2013). The haplotype consists of four coding SNPs in strong linkage disequilibrium (LD), one of which, rs10880, was independently associated with age at LoA. Homozygotes for the minor allele T at rs10880 (T1140M), in LD with the haplotype IAAM, showed later LoA. The proposed mechanism is that the“IAAM”protein isoform results in a more stable latent TGβcomplex, reducing TGFβ signaling and its pro-fibrotic downstream effects (Ceco and McNally 2013; Ceco et al. 2014).

In this study we focused on dilated cardiomyopathy (DCM), a significant clinical feature of DMD. Even if DCM onset and progression are variable, by the end of the second decade, most patients exhibit cardiac insufficiency (McNally et al. 2015; Nigro et al. 1990; Spurney 2011). Currently, glucocorticoid corticosteroids are the main available pharmacological therapy for DMD, but there are contradictory reports on their effect on cardiac function (Markham et al. 2008; Silversides et al. 2003; Barber et al. 2013; Ashwath et al. 2014; Spurney et al. 2014).

In collaboration with a network of Italian neuromuscular Centers, we collected data regarding the onset of dilated cardiomyopathy, defined as the age at first presentation of echocardiographic alterations (left ventricular ejection fraction < 50% or telediastolic volume > 70 mL/m^2), in a cohort of 178 DMD patients. I genotyped this cohort at *SPP1* rs28357094 and the full *LTBP4* haplotype (consisting of 4 SNPs), using TaqMan assays and end-point allelic discrimination on an ABI-7000 SDS instrument. In the determination of LTBP4 haplotype, genotype at the fourth SNP rs1051303

(T820A) was imputed from rs1131620 genotype, assuming no recombination events due to strong LD between these two SNPs which are in very close physical proximity.

Our aim was to test the hypothesis that *LTBP4* and/or *SPP1* genotypes precipitate or delay the onset of cardiomyopathy in DMD, alone or in concurrence with glucocorticoid therapy.

Our results,did not support the effectiveness of steroid treatment in delaying DCM. In fact patients who never developed DCM, or developed it late in life, tended to be more frequently untreated. This may suggest a detrimental effect of glucocorticoids on the myocardium as observed in *mdx* mice (Bauer et al. 2009; Sali et al. 2012), although the prevailing hypothesis in the literature is that glucocorticoids do indeed delay the onset and progression of DCM (Markham et al. 2008).

The *SPP1* rs28357094 SNP and the LTBP4 VTTT/IAAM haplotype were successfully genotyped in the whole cohort for *SPP1* and in 93~94% of the cohort for *LTBP4*, because of limited DNA availability. Results are summarized in Table 1.

	SPP1 rs28357094 (T>G)	*LTBP4* rs2303729 (G>A)	*LTBP4* rs1131620 (A>G)	*LTBP4* rs10880 (C>T)
Major allele homozygote	111 (62.4%)	68 (41.0%)	61 (36.5%)	68 (40.5%)
Heterozygote	59 (33.1%)	66 (39.8%)	67 (40.1%)	69 (41.1%)
Minor allele homozygote	8 (4.5%)	32 (19.2%)	39 (23.4%)	31 (18.4%)
Total	178 (100%)	166 (100%)	167 (100%)	168 (100%)
Minor allele frequency (MAF)	21.1%	39.2%	43.4%	39.0%
Hardy-Weinberg equilibrium (χ^2 p-value)	n.s.	0.035	0.018	0.07

Note: totals do not coincide exactly because of limited DNA availability in a few individuals.

Two *LTBP4* SNPs showed a significant deviation from Hardy-Weinberg equilibrium. Genotyping quality was checked in duplicate experiments. This may be due to population substructure, i.e. participant groups from different regions of Italy having different haplotype structures and MAFs.

We observed an association of later median onset of cardiomyopathy with the dominant G allele at SPP1 rs28357094 and the recessive T genotype at *LTBP4* rs10880, although the log-rank test comparing age at DCM onset between genotype groups, in a time-to-event statistical model, was not significant. This trend was clearer for the rs10880 than the other SNPs and apparently stronger for the homozygous IAAM haplotype, despite its rarity in the studied population (9.6%). This suggests a functional effect of the T1140M aminoacid change within the TGFβ-binding domain of LTBP4. In the literature the association of LoA in DMD with SNPs is suggested to depend on steroid treatment, as observed for *SPP1* (Bello et al. 2012) and for *LTBP4* (Flanigan et al. 2013). Therefore, we limited our analyses of DCM-free survival to 73 steroid-treated patients, and we observed a statistically significant delay of DCM onset in association to the rs10880 T/T genotype. Median ages at DCM onset associated to rs28357094 and rs10880 genotypes is summarized in Table 2.

		***SPP1* rs28357094**		***LTBP4* rs10880**	
	All patients	**T/T**	**T/G-G/G**	**C/C-C/T**	**T/T**
Median age at DCM onset	20.0 years (n = 178)	19.1 years (n = 111)	24.1 years (n = 67)	19.0 years (n = 137)	29.5 years (n = 31)
Median age at DCM onset in steroid treated	20.0 years (n = 75)	17.0 years (n = 45)	24.0 years (n = 30)	***17.9 years (n = 60)*** *	***< 50% DCM[§] (n = 13)*** *
Median age at DCM onset in untreated	20.5 years (n = 88)	20.1 years (n = 54)	20.2 years (n = 34)	20.2 years (n = 65)	25.8 years (n = 15)

Table 2: Median age at DCM onset by *SPP1* and *LTBP4* genotype. *Significant difference between genotypes (log-rank p<0.05).

The most interesting finding appears the apparent preservation from cardiomyopathy of patients with the homozygous rs10880 T genotype and/or IAAM, in concurrence with steroid treatment. If steroids activate a pro-fibrotic cascade thrrough the mineralocorticoid receptor, these patients might be preserved by steroid-induced cardiac damage because they retain TGFβ in the matrix within the stabilized latent complex.

LoA associated to *SPP1* genotype was studied in 123 participants who had available ambulation history (as the main focus of the study was cardiological) and had not been included in our previous reports (Pegoraro et al. 2011). In these, the original association was not validated. Moreover, quite surprisingly, and opposite to what observed in other studies (Flanigan et al. 2013; Bello et al. 2015; van den Bergen et al. 2015a), we observed that the homozygote IAAM in LTBP4 haplotype was associated with significantly *earlier* LoA, and not later LoA. These discrepancies could be explained by the retrospective nature of the cohort used for the analyses (average year of birth 1990), as

well as the heterogeneity in clinical features and standards of care. At that time, steroid therapy was not an universal standard of care. In fact, the young median age of LoA (earlier than 11 years old) might reduce statistical power for validation of LoA associations in this cohort.
Lastly, we did not observe a significant correlation between LoA and DCM onset. This support the hypothesis that skeletal muscle and myocardic dystropathology follow independent progression in DMD and that different genetic modifiers could act in a tissue dependent manner and in a diverse time-frame within the same disease.
These findings are part of the article: "Genetic Modifiers of Duchenne Muscular Dystrophy and Dilated Cardiomyopathy" (Barp et al. 2015), of which I was a co-author, having been responsible for DNA handling, quality control, genotyping, and association of genotypes with clinical data for samples from all participating Centers, which were sent to our laboratory.

The second modifier has been identified thanks to a genome-wide association study designed to discover genetic associations with age of LoA in the CINRG Duchenne Natural History Study (Bello et al. 2016). Genotyping with the Illumina HumanExome chip was performed in 175/340 CINRG-DNHS participants who had sufficient DNA available. Data cleaning was performed by PLINK and included the following: missing-call thresholds of 0.01 for both samples and SNP assays, in order to remove faulty genotyping assays and low-quality DNA samples that might lead to false positive or flase negative findings; a heterozygosity threshold of ±4 standard deviations from the mean, in order to avoid the inclusion of contaminated samples; and a check for cryptic duplicates and relatedness in an IBS matrix (PIHAT threshold of 0.1), in order to prevent stratification biases due to labeling errors and unrecognized relatedness between participants. A subcohort of 109 unrelated individuals of European or European American descentwas selected by multidimensional scaling (MDS) analysis of exome-chip genotypes. This technique allows to select a sub-cohort of relatively homogeneous ancestry, preventing bias and false positive findings deriving from population substructure. Repeated MDS showed no relevant population stratification in theselected subcohort. The exome chip is focused on functional (coding or regulatory) variants within or close to gene-coding regions, and it was selected during study design with the purpose of enhancing GWAS power by reducing the number of studied SNPs and thus of parallel tests, as well as concentrating on functional SNPs (i.e. regulatory or protein-altering). The phenotype was age at loss of ambulation as a time-to-event variable, with glucocorticoid use (before LoA) as a

binary covariate (treated at least 1 year vs. less or untreated). While the main GWAS experiment was negative, as no SNP surpassed the exome-wide significance threshold of $1.8*10^{-6}$, data was re-analyzed concentrating on 438 SNPs within genes involved in pro-inflammatory (NF-κB related) and pro-fibrotic (TGFβ related) pathways, given the recognized role of these mechanisms in driving dystrophin-deficient muscle pathology. The main hit was in *CD40*, also known as tumor necrosis factor superfamily member 5, which encodes a co-stimulatory protein involved in T helper cell polarization,found on the surface of antigen-presenting cells. The T allele at rs1883832 (C>T), adjacent to the translation start ATG codon in the *CD40* 5' UTR, disrupts a translationally relevant Kozak sequence, and a SNP in the promoter region, in very strong LD (rs6074022), seems to reduce *CD40* transcriptional activity evaluated in whole-blood mRNA (Gandhi et al. 2010). Furthermore, the minor haplotype at this locus has been associated to increased alternative splicing of Δ-exon-6-secreted isoform, which might act as a decoy receptor (Onouchi et al. 2012), probably because of LD with other SNPs. rs1883832 has been repeatedly identified as a hit by GWAS studies for the risk of several inflammatory diseases (Jacobson et al. 2005; Onouchi et al. 2012; Gandhi et al. 2010), and T-cell signaling has been established as a relevant pathological event in the secondary inflammation of dystrophin-deficient muscle (Rosenberg et al. 2015; Gussoni et al. 1994; Morrison et al. 2000; Morrison et al. 2005; Farini et al. 2007; Cascabulho et al. 2012; Villalta et al. 2014; Kissel et al. 1991).

The identification of *CD40* as a possible candidate gene implicated in DMD progression was reached on a sub-cohort of European or European-American ancestry (109 patients). Validation studied was subsequently expanded on a very large (for DMD research standards) independent cohort of 660 DMD patients. This validation cohort resulted from the sum of several different clinical cohorts: a secondary CINRG-DNHS cohort (n=76), consisting of participants whose DNA had not been sufficient for exome chip studies, but was available for targeted genotyping; the European Bio-NMD DMD cohort, in which the modifier effect of LTBP4 had been validated (van den Bergen et al. 2015b); the United Dystrophinopathy Project cohort (n=254), in which *LTBP4* was first identified as a modifier (Flanigan et al. 2013); and our retrospective DMD cohort at the University of Padova (n=95), in which the first modifier *SPP1* had been shown to determine age at loss of ambulation (Pegoraro et al. 2011).

I was responsible for the genotyping of the Padova cohort using a TaqMan allele discrimination assay. Genotypes were as follows: CC 47, CT 40, TT 8 (MAF 29%, close to expected in a Caucasian population, no deviation from HWE), with corresponding ages of median loss of ambulation of

11.0, 10.8, and 10.2 years. While the effect of the SNP was not significant in the isolated Padova cohort, the difference in age at LoA was in the same direction, so that the Padova cohort contributed to the independent validation of the association within the unified validation cohort of 660 patients.

Taken together, these findings confirm that patients carrying the T allele at rs1883832 lose ambulation earlier than patients carrying the C allele. In order to study the functional role of this SNP in DMD, we hypothesized that the rs1883832, and more in general the rs6074022-rs1883832 haplotype, may influence the expression of CD40 through transcriptional or post-transcriptional mechanisms. In order to begin to characterize these mechanisms, I performed RT-PCR and immunoblot for the CD40 transcript and protein in DMD muscle biopsy samples from our Center's Biobank. RT-PCR was performed in 16 DMD muscle biopsies from individuals with known rs1883832 genotype (4CC, 8CT and 4 TT). The T allele, in a dominant model, was associated with significantly higher levels of *CD40* transcript (p=0.005) (Figure 1A) and trended to a lower Δ-exon-6 alternatively spliced transcript (p=0007). We performed immunoblot quantification of CD40 protein in 6 muscle biopsy samples (3CC and 3 TT). The small number of samples used for immunoblot limited the ability to perform a statistically significant comparison, but CD40 protein tend to be lower in TT than CC patients (Figure 1B).

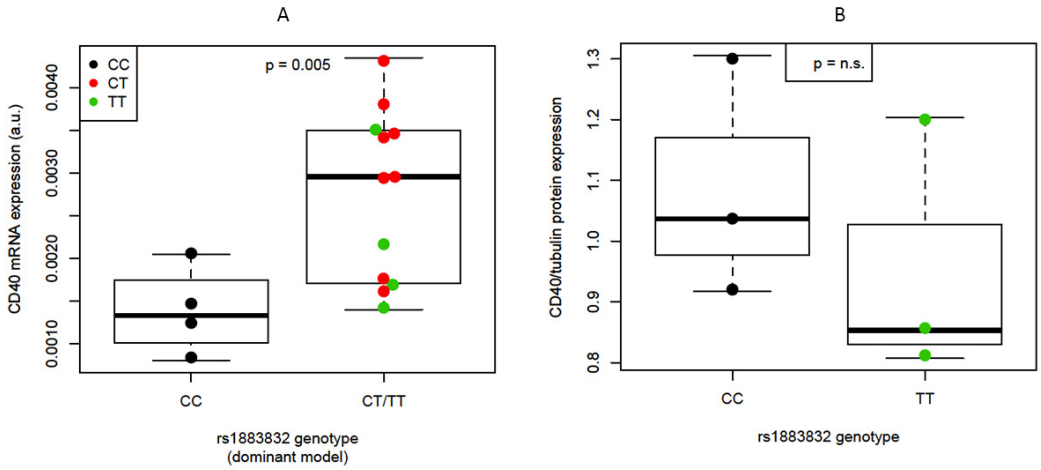


Figure 1: (A) CD40 mRNA expression assessed by rtPCR in the muscle biopsy tissue of DMD patients of given rs1883832 genotypes. (B) Quantification of CD40 protein by Western blot (expressed as a ratio to tubulin) in the muscle biopsy tissue of 3 CC vs. 3 TT DMD patients.

The results obtained with mRNA analysis were opposite to what previously found in whole-blood-derived mRNA (Gandhi et al. 2010), while the protein data was in the same direction as previously published (Jacobson et al. 2005). Overall, these results suggest a complex effect of rs1883832 at both transcriptional and translational levels, involving tissue-specific and disease-specific mechanisms. In conclusion, reduced CD40-mediated cell-cell signaling in carriers of the minor rs1883832 allele might precipitate failure of regeneration and fibrosis in DMD skeletal muscle.

The implication of CD40 and its ligand (CD40L) in fibrosis has been well characterized in renal disease and transplant where CD40/CD40L is a key mediator in inflammation and fibrosis through an increase expression of proinflammatory mediators (Haller et al. 2017; Pontrelli et al. 2006; Kraus et al. 2013). In order to better understand the active role of CD40 and the carrier of the minor allele at rs1883832, it would be useful to study possible inflammatory targets of CD40 and how they modulate pro-fibrotic pathways, in particular on the connective tissue growth factor CTGF/CCN-2 that stimulates procollagen and fibronectin production (G. Sun et al. 2008; Morales et al. 2017)

Materials and methods

All patients were genotyped by TaqMan essays and end-point allelic discrimination on an ABI-7000 SDS instrument. For more details see (Bello et al. 2016).

These results are part of the article: “Association Study of Exon Variants in the NF-kB and TGFb Pathways Identifies CD40 as a Modifier of Duchenne Muscular Dystrophy” (Bello et al. 2016).

Conclusion

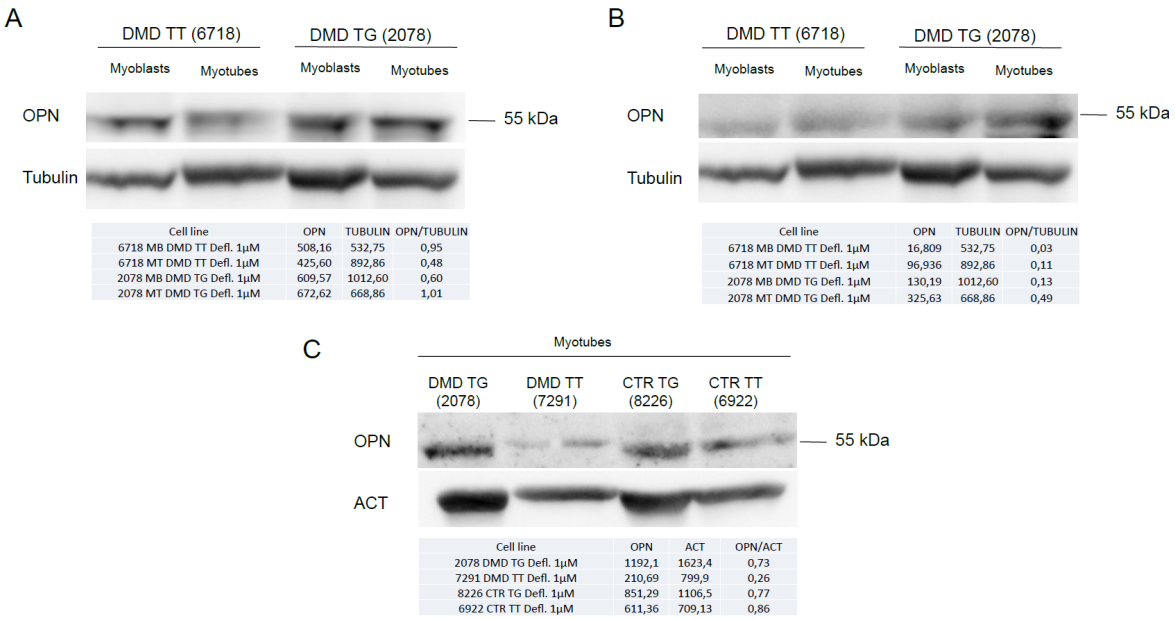
Numerous relevant and innovative aspects of the molecular mechanisms underlying genetic modifier effects in DMD have been investigated in my PhD project. The findings presented in this thesis illustrate the relevant impact of genetic modifiers on inter-patient variability in DMD. In particular, the genetic background of osteopontin at rs28357094 not only determinates the age of loss of ambulation, as patients carrying the TT genotype lose ambulation later than TG (Pegoraro et al. 2011; Bello et al. 2015), but also the response to glucocorticoid treatment (Vianello et al. 2017). Our hypothesis of interaction between GRE element and deflazacort is validated in myoblasts, where *SPP1* mRNA levels are higher in the TG than TT genotype. Moreover a multivariate analysis, taking in account the concurrent effect of dystrophin deficiency and rs28357094 genotype, showed a significant interaction, further suggesting an implication of GREs. On the other hand, results on protein expression underline the complexity of post-transcriptional and post-translational regulation of *SPP1* expression (Pagel et al. 2014).

Our study of the isoforms of OPN confirms that all three isoforms are expressed in DMD muscle, with a predominance of OPNb, and a tendency of all three isoforms to reduction with increasing age, as active inflammation gives way to end-stage fibrosis. Further studies are needed to better elucidated isoform-specific pathogenic mechanisms.

Genetic modifiers were initially associated to the age of loss of ambulation, but our results on *LTBP4* and *SPP1* indicate that they are implicated in the age of onset of cardiomyopathy too, a major complication and cause of death in DMD. Both genetic modifiers delayed the onset of the signs of DCM by several years, about 10 for *LTBP4* and 5 years for *SPP1.*

Finally, my PhD work contributed to the validation of a novel genetic modifier, CD40. Our findings allow a better comprehension of the role of the genetic modifiers in DMD, and have potentially relevant implications for the selection of homogeneous groups of patients for clinical trials, for a deeper knowledge of the pathophysiology of dystrophin deficiency, and for planning novel therapeutic strategies.

Supplementary figures of Aim 1:

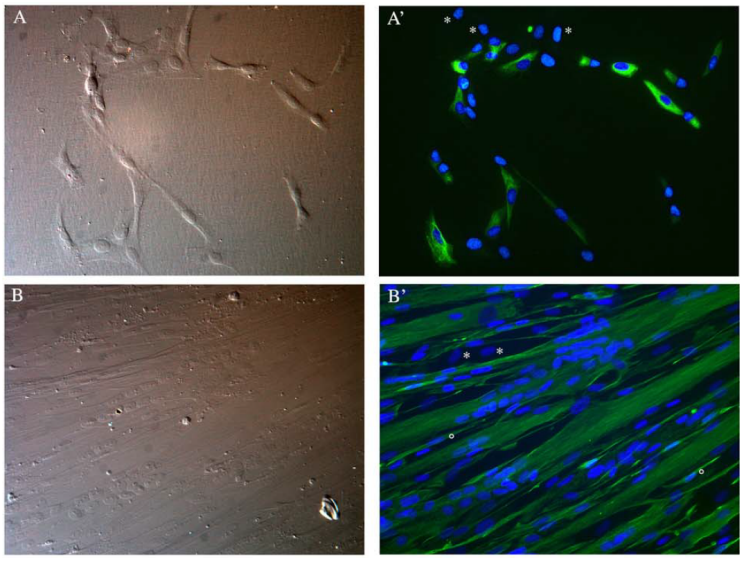


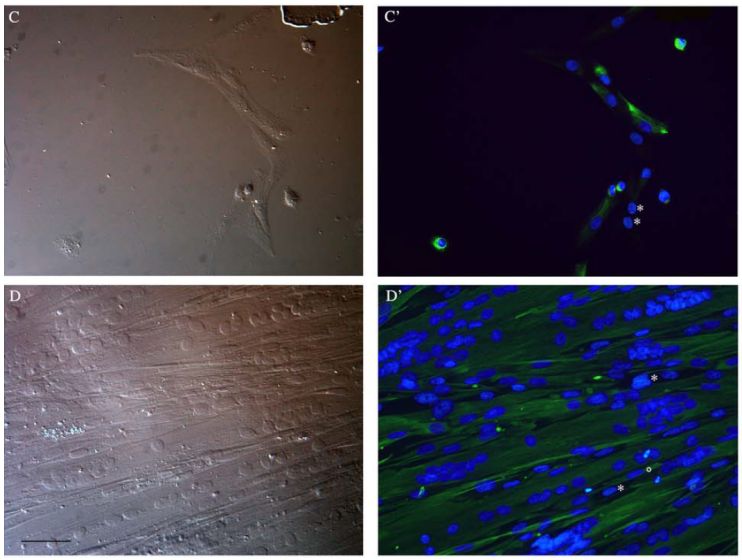
Cell line	OPN	TUBULIN	OPN/TUBULIN
6718 MB DMD TT Defl. 1µM	508,16	532,75	0,95
6718 MT DMD TT Defl. 1µM	425,60	892,86	0,48
2078 MB DMD TG Defl. 1µM	609,57	1012,60	0,60
2078 MT DMD TG Defl. 1µM	672,62	668,86	1,01

Cell line	OPN	TUBULIN	OPN/TUBULIN
6718 MB DMD TT Defl. 1µM	16,809	532,75	0,03
6718 MT DMD TT Defl. 1µM	96,936	892,86	0,11
2078 MB DMD TG Defl. 1µM	130,19	1012,60	0,13
2078 MT DMD TG Defl. 1µM	325,63	668,86	0,49

Cell line	OPN	ACT	OPN/ACT
2078 DMD TG Defl. 1µM	1192,1	1623,4	0,73
7291 DMD TT Defl. 1µM	210,69	799,9	0,26
8226 CTR TG Defl. 1µM	851,29	1106,5	0,77
6922 CTR TT Defl. 1µM	611,36	709,13	0,86

Supplementary Figure 1. Western Blots (WB) of protein lysates from DMD and control muscle cells treated with Deflazacort, using two alternative OPN antibodies. Quantitation data by optical densitometry are shown below each panel. (A) WB using the ab8448 Abcam Rabbit polyclonal anti-OPN antibody raised against the CKSKKFRRPDIQYPD peptide (aa 170-183 of human OPN), with tubulin as loading control, in two cell lines (both myoblasts and myotubes). (B) WB using the AKm2A1 (sc-21742) Santa Cruz Mouse monoclonal antibody against recombinant murine OPN, with tubulin as loading control, in two DMD cell lines (both myoblasts and myotubes). (C) WB using the AKm2A1 (sc-21742) Santa Cruz Mouse monoclonal antibody against recombinant murine OPN, with actin as loading control, in two DMD and two control myotube cultures. In all experiments, OPN expression was higher in TG than TT myotubes, in line with our findings with the main antibody used in this paper.





Supplementary Figure 2. Upper panel: examples of control myoblasts (A) and myotubes (B). Lower panel: examples of DMD myoblasts (C) and myotubes (D). Images to the left were acquired by Nomarski differential interference contrast; images to the right were obtained by immunofluorescence microscopy, after staining with a monoclonal anti-desmin antibody (green) and DAPI (blue). Images A' and C' show that amongst myoblasts the levels of desmin expression could differ widely amongst single cells, something that in our experience is the norm in biopsy-derived primary cultures, especially when derived from DMD muscle. Images A' and C' also show that after CD56 enrichment cultures from non-dystrophic muscle still comprised a sizeable non-myogenic fraction (most likely fibroblasts). Such cells, which in our hands averaged around 10% in control preps, remained desmin-negative even upon gross overexposure; in DMD preps the percentage of desmin-negative cells was usually higher. Examples of such cells are indicated by asterisks. Images C' and D' show how both control and DMD myoblasts yielded very large myotubes upon differentiation, with only few single myoblasts (examples of which are labeled with circles). As opposed to what was seen in myoblasts, desmin expression was homogeneous across differentiated cultures. Immunostaining was performed by incubating cells overnight at 4°C with Dako anti-desmin antibody (clone D33, M0760) diluted 1:50 in PBS-3% BSA. Secondary antibody was an AF488-conjugated anti-mouse from Jackson Immunoresearch, diluted 1:200 and incubated for one hour at RT.

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