Università degli Studi di Padova

Head Office: Università degli Studi di Padova

Department of Cardiac, Thoracic, Vascular Sciences and Public Health

Ph.D. COURSE IN: Medicina traslazionale “G. B. Morgagni”
CURRICULUM: Neuroscience
SERIES: XXXII

# *GENETIC AND CLINICAL MODIFIERS IN DUCHENNE MUSCULAR DYSTROPHY*

Thesis written with the financial contribution of Fondazione Cariparo

**Coordinator:** Prof. Annalisa Angelini
**Supervisor**: Prof. Elena Pegoraro

**Ph.D. student**: Aurora Fusto

# Table of Contents

# Acronyms

2D: two-dimensional

3D: three-dimensional

*ACTN2*: α-actinin-2

*ACTN3*: α-actinin 3

*Actn3*: *ACTN3* orthologous in mouse

ANCOVA: analysis of covariance

APC: antigen presenting cell

BMD: Becker muscular dystrophy

$Ca^{2+}$: calcium ion

*CD40*: cluster of differentiation 40

CD40L: CD40 ligand

CCD: central core disease

Cdk4: cyclin-dependent kinase 4

CI: confidence interval

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

CTCF: CCCTC-binding factor

DAMP: damage-associated molecular pattern

DCM: *DMD*-associated dilated cardiomyopathy

DCM: dilated cardiomyopathy

Defl: deflazacort

DFZ: deflazacort

DGC: dystrophin-glycoprotein complex

DHPR: dihydropyridine receptor

DMD: Duchenne muscular dystrophy

*DMD*: dystrophin gene

*dmm:* double-mutant *Spp1* negative *mdx* mouse

Dp427B: dystrophin brain isoform

Dp427M: dystrophin muscular isoform

Dp427P: dystrophin Purkinje cerebellar neurons isoform

ECC: excitation-contraction coupling

ECM: extracellular matrix

EDV: end-diastolic volume

EF: ejection fraction

FEV1: forced expiratory volume in 1 second

FS: frameshift

FS: fractional shortening

FVC: forced vital capacity

FU: follow-up

GC: glucocorticoid corticosteroid

GEE: generalized estimating equations

GWAS: genome-wide association studies

GRE: glucocorticoid response element

IHC: immunohistochemistry

HR: hazard ratio

hMMT: human skeletal muscle micro-tissue

hTERT: human telomerase reverse transcriptase

HWE: Hardy-Weinberg Equilibrium

LD: linkage disequilibrium

LoA: loss of ambulation

*LTBP4*: latent transforming growth factor β binding protein 4

LV: left ventricle

MAF: minor allele frequency

*mdx:* X-linked muscular dystrophy mouse

MHCI: class I major histocompatibility complex

MHCII: class II major histocompatibility complex

MW: molecular weight

mRNA: messenger RNA

NF-κB: nuclear factor κB

NIV: non-invasive ventilation

OPN: osteopontin

ORF: open-reading-frame

PCR: polymerase chain reaction

PEF: peak expiratory flow

PFT: pulmonary function test

PRED: prednisolone

PUL: performance of upper limb

RT-PCR: real-time PCR

RyR1: ryanodine receptor 1

*RYR1*: ryanodine receptor 1 gene

SD: standard deviation

SE: standard error

SERCA: sarcoplasmic reticulum $Ca^{2+}$-ATPase

siRNA: silencing RNA

SNP: single nucleotide polymorphism

*SPP1*: secreted phosphoprotein 1 gene

*Spp1: SPP1* orthologous in mouse

SR: sarcoplasmic reticulum

TGFβ: transforming growth factor β

*THBS1*: thrombospondin-1

TLR: toll-like receptor

*TNFRSF5*: tumour necrosis factor receptor superfamily member 5

UDP: United Dystrophinopathies Project

WB: western blot

# Riassunto

La distrofia muscolare di Duchenne (DMD) è una malattia neuromuscolare causata da mutazioni del gene codificante per la distrofina (*DMD*) che ne impediscono la produzione. Sebbene tutti i pazienti affetti da DMD condividano lo stesso difetto biochimico di distofina, a livello fenotipico è osservabile una grande varietà in termini di progressione della malattia, ad esempio nell'età di perdita della deambulazione o nell'età di insorgenza di complicanze cardiache e respiratorie. Questa variabilità è dovuta a diversi fattori, alcuni di origine ambientale (ad esempio la qualità delle cure a cui hanno accesso i malati) e altri di natura genetica, suddivisibili in *cis-acting*, ossia l'effetto dei diversi tipi di mutazioni nel gene *DMD* sul fenotipo, e *trans-acting*, ovvero l'effetto di SNPs modificatori sul fenotipo. Questi ultimi sono polimorfismi in geni diversi da quello causativo della malattia, che hanno però un effetto sul suo fenotipo. Usando come outcome la perdita della deambulazione sono stati individuati numerosi SNPs modificatori, quali: rs28359074 in *SPP1,* rs2303729, rs1131620, rs1051303 e rs10880 in *LTBP4,* rs1883832 e rs6074022 in *CD40*, rs1815739 in *ACTN3,* rs2725797 and rs2624259 in *THBS1*.

L'obiettivo del mio percorso di dottorato è stato lo studio della variabilità genetica e clinica nella distrofia muscolare di Duchenne, conducendo indagini *in vitro* e studi osservazionali retrospettivi.

Il primo approccio è stato utilizzato per verificare l'interazione dello SNP modificatore rs28357094 nel gene *SPP1*, codificante la proteina osteopontina (OPN), e il trattamento farmacologico con glucocorticoidi (nello specifico deflazacort) in mioblasti e miotubi primari derivati da controlli sani e da pazienti DMD. Lo studio ha messo in evidenza che l'osteopontina è sovraespressa in miotubi con genotipo TG per lo SNP rs28357094, rispetto a TT. Inoltre, è stato rilevato che il trattamento con Deflazacort induce l'aumento della produzione di OPN solo nei miotubi con genotipo TG. Questi risultati hanno confermato l'interazione tra il modificatore genetico e il trattamento con glucocorticoidi, sottolineando l'importanza del genotipo di rs28357094 nella risposta al trattamento farmacologico nei pazienti DMD.

Successivamente, il nostro interesse si è rivolto allo studio dell'effetto non solo degli SNPs modificatori, ma anche dell'effetto delle diverse mutazioni nel gene della distrofina (*DMD*) e del trattamento farmacologico sul decorso della malattia nei pazienti DMD, focalizzando la nostra attenzione su diversi aspetti fenotipici, quali: la performance degli arti superiori, la funzione respiratoria e cardiaca. L'obiettivo di questi studi, resi possibili dalla collaborazione di numerosi centri italiani nella raccolta dei dati clinici, è stato quello di evidenziare potenziali nuovi target terapeutici e di fornire importanti informazioni per la stratificazione dei pazienti nel corso dei trial clinici.

Il nostro lavoro ha permesso di confermare l'influenza di alcuni degli SNPs, noti per il loro effetto sulla perdita della deambulazione, anche su altri parametri clinici consentendoci di identificare misure di efficacia clinica nella DMD. È stato poi possibile documentare l'effetto protettivo del trattamento con

glucocorticoidi anche su aspetti della malattia non strettamente correlati alla deambulazione, come la funzionalità respiratoria e cardiaca e dimostrare come alcune mutazioni nel gene *DMD* abbiano effetti diversi sull'espressione del fenotipo dei pazienti.

Infine, il mio interesse si è rivolto al *modelling* di malattie neuromuscolari in sistemi di coltura tridimensionali, con lo scopo di far luce sui meccanismi molecolari causativi e fornire piattaforme utili per la ricerca e il test di molecole con azione farmacologica.

# Abstract

Duchenne muscular dystrophy (DMD) is a neuromuscular disease caused by out-of-frame mutations in the *DMD* gene resulting in the lack of dystrophin in skeletal muscle fibres. Even though all DMD patients share the same molecular defect, it is possible to observe high variability in the disease's progression, i.e. differences in loss of ambulation age, onset of respiratory and cardiac failure.

This variability is due both to environmental and genetic factors. Genetic factors may be divided in *cis-acting*, nominally the type of *DMD* mutation, and *trans-acting*, or modifier SNPs. These are polymorphisms in genes, different from the causative *DMD*, that have and effect on the phenotype. There are several modifier SNPs known to alter age at loss of ambulation. These are: rs28359074 in *SPP1,* rs2303729, rs1131620, rs1051303 e rs10880 in *LTBP4,* rs1883832 e rs6074022 in *CD40*, rs1815739 in *ACTN3,* rs2725797 e rs2624259 in *THBS1*.

The main goal of my PhD was the study of clinical and genetic variability in DMD, through *in vitro* and observational retrospective studies.

We carried an *in vitro* research to verify the interaction of rs28357094 in *SPP1*, that codifies for osteopontin (OPN), and glucocorticoids treatment (Deflazacort) in primary myoblasts and myotubes derived from healthy individuals and DMD patients. We found that OPN is overexpressed in rs28357094 TG genotype myotubes, compare to TT genotype. Moreover, deflazacort treatment induces an increase in OPN production in TG myotubes. These results confirmed the interaction between rs28357094 and glucocorticoids treatment.

Afterwards, we studied the effect of the known modifiers, on multiple phenotypic aspects: upper limbs performance, respiratory and cardiac function. These analyses had been made possible thanks to the collaboration in the data collection phase of several Italian centres. Our goals were to find new potential therapeutic targets and to provide information useful for patients stratification in clinical trials.

We were able to confirm the effect of some SNPs, known to be modifier of age at loss of ambulation, on diverse outcomes measures as performance of upper limbs, respiratory and cardiac function. Furthermore, we assess the protective effect of glucocorticoids treatments on diseases aspects other than ambulation, and provide new information about the correlation between *DMD* mutations and phenotype severity.

Finally, I switched my interest to three-dimensional modelling of neuromuscular diseases, aiming to clarify pathological mechanisms and provide a versatile platform for drug screening and test.

# Introduction

## Skeletal muscle and dystrophin

The skeletal muscle is a highly organized tissue, which comprises muscle fibres and connective tissue. Each muscle fibre is a multinucleated post-mitotic cell, surrounded by the plasma membrane and basal lamina, the sarcolemma (Frontera and Ochala, 2015; Victor Dubowitz and Sewry, 2007). Since myonuclei are post-mitotic, they are unable to contribute to skeletal muscle growth and regeneration, satellite cells - the skeletal muscle stem cells, are involved in these processes (Chang and Rudnicki, 2014; Yin et al., 2013).

The functional unit of skeletal muscle is the sarcomere, which shortening is at the base of muscle contraction. In healthy skeletal muscle, an action potential from the motor neuron triggers acetylcholine release at the neuromuscular junction which induces an action potential that travels along the sarcolemma (Frontera and Ochala, 2015). This structure penetrates deeply into the myofiber in repeating structures called T-tubules. T-tubules form the triad together with two terminal cisternae of the sarcoplasmic reticulum (SR), the main calcium storage region in skeletal muscle. The triad is central to excitation-contraction coupling (ECC), the process by which an action potential triggers the synchronous contraction of the myofibrils, which are made up of aligned sarcomeric units. During ECC, the action potential is propagated along the sarcolemma and the T-tubule to the triad. Here, a voltage sensor subunit of the dihydropyridine receptor (DHPR, also known as $Ca_v1.1$) changes conformation, and triggers the opening of the ryanodine receptor 1 (RyR1) in the terminal cisternae of the SR, to which it is mechanically coupled. RyR1 releases large amounts of calcium ions ($Ca^{2+}$) into the sarcoplasm of the muscle fibre (Frontera and Ochala, 2015; Rebbeck et al., 2014), where it interacts with the repeating contractile units of the myofibrils. $Ca^{2+}$ binds to troponin which triggers the reconfiguration of the actin–tropomyosin structure that exposes myosin binding sites and allows myosin heads to bind to actin via crosslinks. Cyclical actin-myosin binding shortens the sarcomere via the sliding filament mechanism, resulting in muscle contraction. Repolarisation of the sarcolemma and T-tubules closes DHPR and RyR1, preventing further $Ca^{2+}$ release. Sarcoplasmic $Ca^{2+}$ is rapidly sequestered into the SR via sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pumps, which enable

the actin–tropomyosin structure to return to its original conformation, blocking myosin head binding and resulting in muscle relaxation (Gomes et al., 2002; Smith et al., 2017).

The integrity of skeletal muscle during contraction is ensured by the skeletal muscle isoform of dystrophin (Dp427M) (Dellorusso et al., 2001; Han et al., 2011; Petrof et al., 2006). This protein is a component of the dystrophin-glycoprotein complex (DGC) and localises in the inner surface of the plasma membrane (Han et al., 2011). Here, its N-terminus interacts with F-actin, while the C-terminus contacts β-dystroglycan. This link is extended to the extracellular matrix (ECM) by α-dystroglycan, which binds to laminin α2, agrin and perlecan with high affinity (Figure 1) (Cohn and Campbell, 2000).

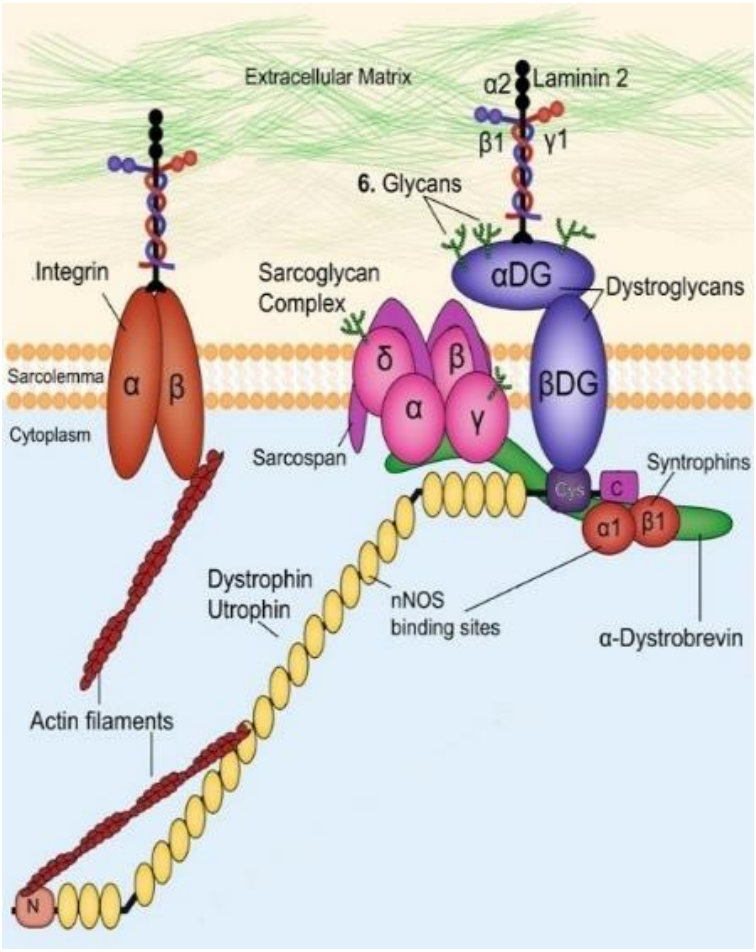


*Figure 1. Components of the dystrophin-glycoprotein complex. Adapted from (Yucel et al., 2018).*

The dystrophin-mediated connection between the cytoskeleton and the ECM stabilises the sarcolemmal structure, transmits force laterally and prevents sarcolemmal damage during contraction (Dellorusso et al., 2001; Han et al., 2011; Petrof et al., 2006). Contraction-induced injury is characterized by two distinct phases: an initial injury and a delayed secondary injury from the inflammatory response. The initial injury consists of mechanical disruption of sarcomeres followed by impaired ECC and $Ca^{2+}$ signalling and finally by activation of $Ca^{2+}$-sensitive degradation pathways

(Jonathan et al., 2005). The damage eventually results in muscle degeneration, necrosis and fibrosis in skeletal muscle (Rando, 2001; Victor Dubowitz and Sewry, 2007).

Dystrophin (DMD) is codified by *DMD*, mutations in this gene lead to the loss of dystrophin or to the production of a truncated protein (Hoffman et al., 1987; Koenig et al., 1987), disrupting the link between cytoskeleton and ECM, leading to sarcolemmal damage upon muscle contraction.

*DMD* is located in the Xp21, it is the largest gene described in humans (2,5 million bp) and codifies for several dystrophin isoforms. There are three full-length isoforms (called Dp427 because of the molecular weight of 427 kDa) made of the same exons, but derived from three independent promoters in brain (Dp427B), muscle (Dp427M), and Purkinje cerebellar neurons (Dp427P).

Dp427M codifies a 14000 bp mRNA, predominantly expressed in skeletal and cardiac muscle with small amounts expressed in the brain (Muntoni et al., 2003; Yaffe et al., 1992). Moreover, *DMD* produces other smaller isoforms through alternative splicing events. The splice variants originate both from exon skipping (exclusion of some exons) and exon scrambling (subverted exons reciprocal order) (Sadoulet-Puccio and Kunkel, 1996). These events usually are tissue-specific and give rise to further

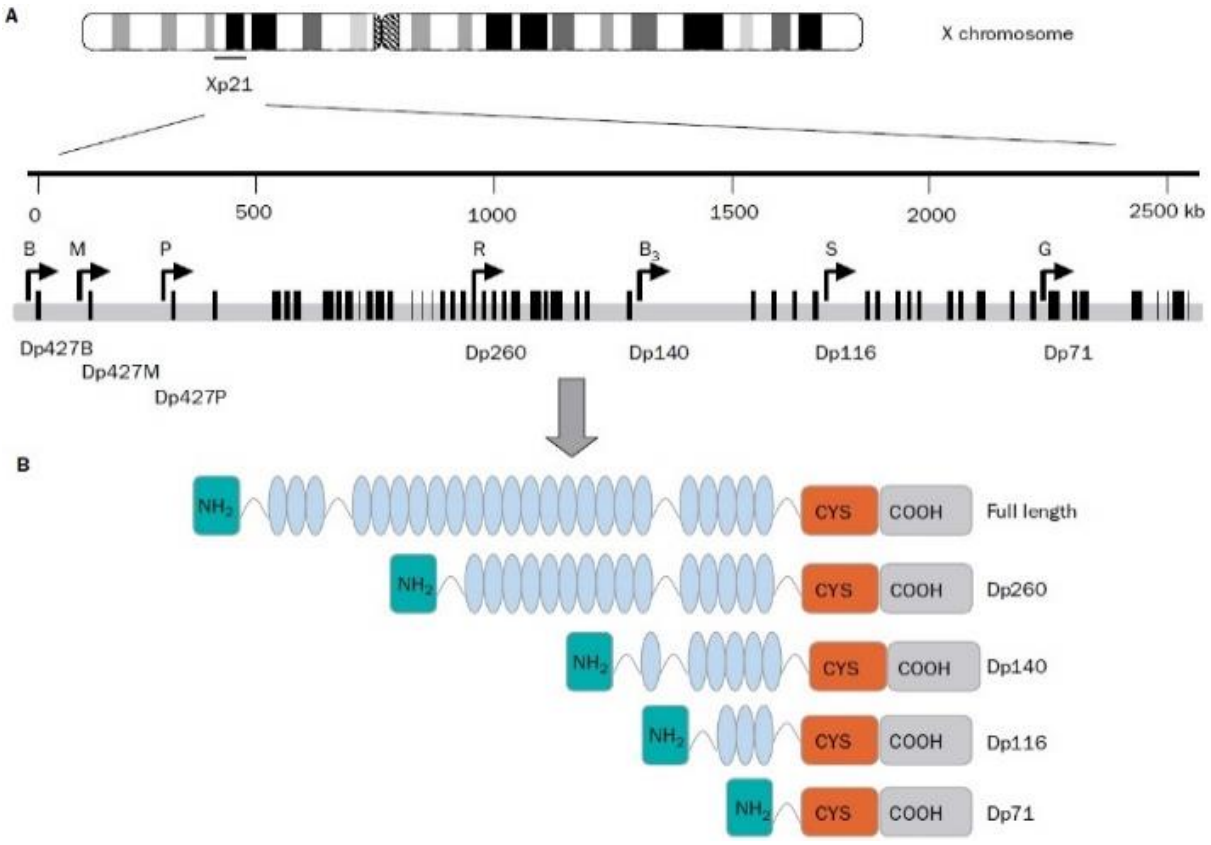


*Figure 2. A) Genomic organisation of the dystrophin gene located in Xp21. The black vertical lines represent the exons of the dystrophin gene. The arrows indicate the various promoters: brain (B), muscle (M), and Purkinje (P) promoters; R, B3, S, and G represent the Dp260 (retinal), Dp140 (brain3), Dp116 (Schwann cells), and Dp71 (general) promoters. B) The domain composition of the various dystrophin proteins is indicated. The amino-terminal domain is followed by the spectrin like domain, the cysteine rich, and the carboxy-terminal domain* (Muntoni et al., 2003).

protein diversity and account for the complex expression regulation of the tissue-specific dystrophin functions (Figure 2) (Muntoni et al., 2003).

## Duchenne Muscular Dystrophy (DMD)

Duchenne Muscular Dystrophy (DMD) had been recognised as clinical entity since nineteenth century. The most famous description of the disease is by GB Duchenne, who detailed depicted 13 patients with progressive muscle weakness (Duchenne GBA, 1868); nevertheless, there are several earlier descriptions by other physicians (Conte and Gioja, 1836; Meryon E, 1852).

The disease affects 1 in 5000 – 10000 male live births (Ryder et al., 2017). DMD, together with Becker Muscular Dystrophy (BMD) and *DMD*-associated dilated cardiomyopathy (DCM), belongs to dystrophinopathies, a spectrum of X-linked muscle disease caused by mutations in *DMD* (Koenig et al., 1987).

Base on the "reading-frame hypothesis", out-of-frame mutations in *DMD* destroy dystrophin open-reading-frame (ORF), resulting in unstable mRNA that leads to nearly undetectable quantities of truncated protein in the plasma membrane of myofibers and cardiomyocytes, this condition causes DMD. On the other hand, when the mutation does not alter the ORF of the transcript, it is possible to detect qualitatively and/or quantitatively altered dystrophin. This type of defect provokes BMD (Koenig et al., 1989). The reading frame hypothesis explains over 90% of cases and is commonly used both as a diagnostic confirmation of dystrophinopathies and for the differential diagnosis of DMD and BMD (Muntoni et al., 2003).

### Clinical history

DMD onset is usually in early childhood with delayed motor milestones including delays in walking independently and standing up from a supine position. Proximal weakness causes difficulty in running, jumping, and standing up from a squatting position (Darras et al., 2018). Weakness of knee and hip extensors results in Gower's manoeuvre to stand (Emery, 2002). DMD progression is rapid, causing affected children to be wheelchair dependent by 12 years of age (Emery, 2002). In the vast majority of cases death is caused by respiratory failure compounded by cardiac involvement, which happens in the late teens or early 20s. Non-muscular symptoms may also be present, they include mental impairment, with about 20% of affected boys have an IQ of less than 70 (Emery, 2002).

Similar to DMD, BMD is characterised by muscle weakness and wasting, but shows delayed onset (around 12 years) and a more benign course of the disease (Emery, 2002). Despite the milder skeletal muscle involvement, heart failure from DCM is a common cause of morbidity and the most common cause of death that usually occurs in the mid-40s for BMD patients (Darras et al., 2018; Emery, 2002).

Finally, DCM is characterized by left ventricular dilation and congestive heart failure. It may affect DMD and BMD patients as well as DMD carriers (females heterozygous for a *DMD* pathogenic variant), in whom DCM can manifest even without apparent weakness (Darras et al., 2018; Emery, 2002), but it may also present in patients without significant muscle weakness.

## Histology

Form a histological point of view, skeletal muscle biopsies from DMD and BMD show rounded fibres, characterised by variety in fibre size, with hypertrophy and atrophy of fibres. Moreover, it is possible to observe necrosis, increased central nuclei, proliferation of endomysial and perimysial connective tissue and increased adipose tissue (Figure 3). These features collectively are often referred as to "dystrophic" and they reflect the progressive loss of muscle and the necrosis of the tissue. Pathological changes can be seen in few months old DMD patients, when there are no clinical manifestations of the disease other than elevated CK. Abnormalities in 1 year old BMD patients can be seen too (Victor Dubowitz and Sewry, 2007).

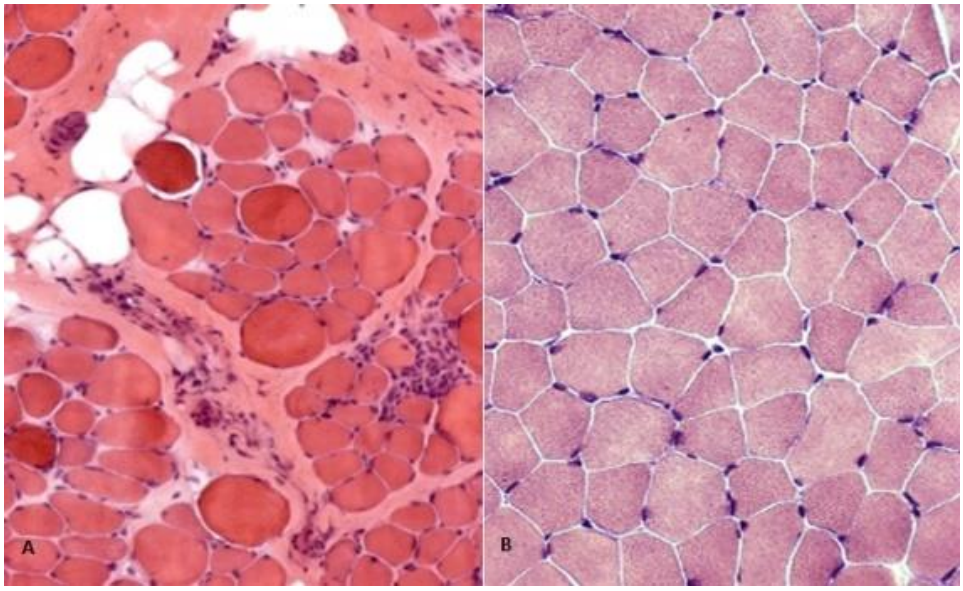


*Figure 3. Haemoxylin-Eosin staining of skeletal muscle biopsy. On the left A) it is possible to appreciate the main histological features of a DMD muscle, such as variation in fibres size, necrosis and fibro-fatty substitution of the tissue. On the right B) a healthy muscle.*

## Pathogenesis

In healthy individuals, skeletal muscle has a relatively low capacity to generate localized immune responses, due to the low number of resident proinflammatory cells. This immune privileged status allows rapid regeneration in healthy muscle, with limited and quick resolved inflammation.

On the contrary, soon after birth, in DMD muscles there is a strong activation of multiple components of the innate immune system, even before the onset of clinical symptoms. The immune activation

includes altered signalling via Toll-like receptors (TLR4, TLR7), via nuclear factor κB (NF-kB), and expression of major histocompatibility complex (MHC) class I molecules on muscle fibres, which are not normally express in healthy muscle. The activation of the immune system is likely mediate by membrane instability, caused by dystrophin lack, and the associated release of cytoplasmic contents into the extracellular space - including DAMPs (damage-associated molecular pattern) molecules, which bind to TLRs with consequent inflammasome formation and the self-sustaining activation of the immune response. Moreover, the microenvironment rich in proinflammatory cytokines induces constitutive MHCI/II expression on muscle cells, the recruitment of T and B cells and the generation of an adaptive immune response in the muscle milieu (Rosenberg et al., 2015).

Upon successive cycles of degeneration, muscle can become necrotic, the following regeneration process takes 2 weeks. In DMD, neighbouring fibres enter the necrotic stage at different times (asynchronous regeneration); this sustain chronic inflammation state, which in turn creates a more proinflammatory environment that activates innate immune response pathways (Figure 4) (Rosenberg et al., 2015).

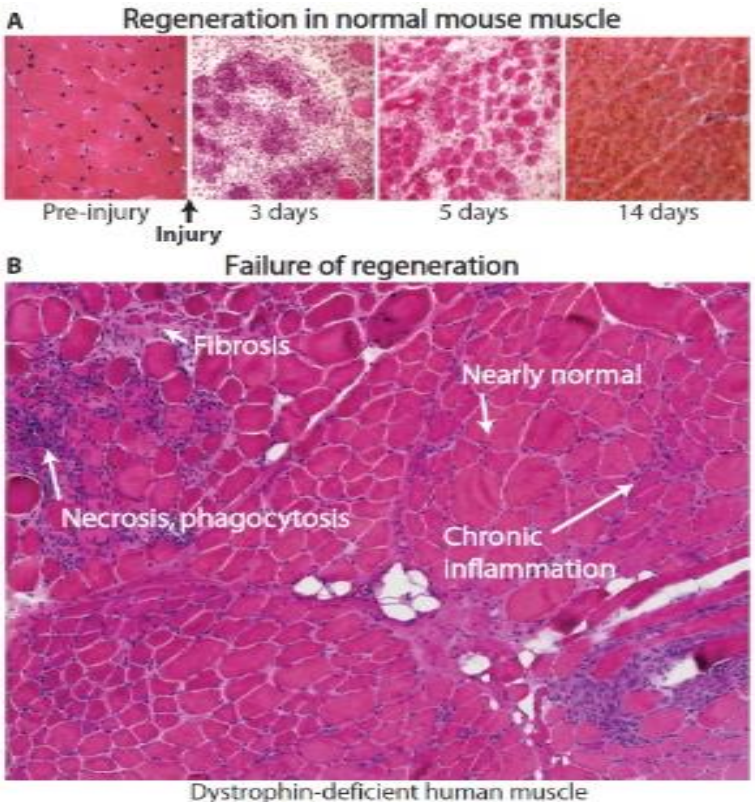


*Figure 4. A) Stages of regeneration in normal mouse muscle after injury. Regeneration of skeletal muscle in response to injury is a highly synchronized process. Within 24 hours of injury, mouse muscle becomes infiltrated with neutrophils. Within 2 to 3 days, the injured muscle is infiltrated by proinflammatory M1 macrophages. During days 5 to 10, the resolution and repair phases of regeneration take place and muscle is predominantly populated by remodelling M2 macrophages. M2 macrophages are essential for complete muscle regeneration, which is achieved by day 14. B) Asynchronous degeneration/regeneration in human dystrophin-deficient muscle. Repair of human dystrophin-deficient muscle after injury is impaired due to asynchronous bouts of degeneration and regeneration, leading to the release of cytokines, such as TGFβ, that initiate and perpetuate fibrosis. Shown is a muscle biopsy from a DMD patient revealing regions of nearly normal myofibers; chronic inflammation (between myofibers); phagocytosis by neutrophils and macrophages, and necrosis; and fibrosis (failed regeneration) (Rosenberg et al., 2015).*

Neutrophils and macrophages are the first cell types to infiltrate the necrotic skeletal muscle, having a major role in phagocyte debris and in stimulation myogenesis during tissue repair (Arnold et al., 2007; Tidball et al., 2014). These cells are also involved in the fibrotic process consequent to the inflammation; in particular, the activation of M2 (modulatory) macrophages is associated with the production of arginase I that is involved in collagen synthesis (Gordon, 2003; Murray et al., 2014; Wynn, 2015), and M1 (inflammatory) macrophages sustain chronic inflammation, and therefore contribute to fibrogenesis as well (Ryu et al., 2009; Suelves et al., 2007).

The most potent fibrogenic factor is transforming growth factor β (TGFβ) (Bowen et al., 2013; Massagué, 2012), which is an important regulatory role in regenerating muscle after injury and is mainly produced by macrophages (Zhou et al., 2006). It is generated as latent precursor, that is stored in the ECM and is activated by tissue damage or specific growth signals (Hinz, 2015; Sterner-Kock et al., 2002; Yan et al., 2015). Activated TGFβ binds to heterodimeric complex comprised of a TGFβ type I receptor and a type II one.

Moreover, TGFβ can induce the fibrogenic conversion of satellite cells (Li et al., 2004; Pessina et al., 2015). These cells produce high level of matrix proteins while losing their identifying gene expression program. These cellular conversions had been proposed too contribute to reduced regeneration capacity of skeletal muscle as well and to the increased fibrosis in *mdx* (Biressi et al., 2014; Pessina et al., 2015).

### Phenotype variability

The classification of dystrophic patients into DMD or BMD is not always easy, and it relies on three orders of evaluations: the characterization of *DMD* mutations at the genomic and transcriptomic level (i.e., prediction of the effect of the mutation on the ORF); protein assays performed on samples obtained from skeletal muscle biopsy, including immunohistochemistry (IHC) and western blot (WB); and, clinical criteria, taking into consideration that the presence of weakness by the age of 5 years, and/or loss of ambulation (LoA) by the age of 13 years are typical for a DMD phenotype, while LoA beyond 16 years is suggestive of BMD (Bello and Pegoraro, 2019).

Within the boundaries of the clinical/molecular definition of DMD, even if all DMD patients carry out-of-frame mutations and lack of full-length dystrophin in skeletal muscle, it is possible to observe a spectrum of phenotype severity (Barp et al., 2015; Humbertclaude et al., 2012; Pane et al., 2014b; Wang et al., 2018). This is primarily measured by age at LoA, because of its impact on daily life and the overall health of patients, and its correlation with overall survival and other disease milestones, such as the onset of respiratory insufficiency, the need for scoliosis surgery (Bello and Pegoraro, 2019).

The phenotype variability is caused by environmental and genetic effects. The genetic effects can be further subdivided in "*cis*" and "*trans*" acting effects.

The effect in "*cis*" are due to the *DMD* mutations themselves, in fact, in DMD patients dystrophin may not always be completely absent from skeletal muscle fibres. Protein assays (IHC and WB) that are commonly used tool in the diagnostic setting have limited sensitivity, so that small amounts of protein may escape detection, while still exerting a measurable effect on the phenotype. (Bello and Pegoraro, 2019).

The "*trans*" acting factors are the genetic modifiers, i.e. polymorphisms in genes - others than the one that causes the disease, which influence disease phenotype affecting onset, progression, response to treatment, etc.

Investigate the phenotype variability in DMD is important because may give a better insight in the disease pathogenesis, pointing out new therapeutic targets. Moreover, an improved understanding of the wide variation that is observed in the trajectories of disease progression among patients would lead to better design of clinical trials (Bello and Pegoraro, 2019).

The study of phenotype variability in DMD, with special focus on genetic modifiers, was the main topic of my PhD and is the subject of this thesis.

### *Environmental effect – standard of care*

The standard care for DMD patients includes physical therapy, management of joint contractures, bone fracture prevention and glucocorticoid corticosteroid (GC) treatment, specifically with prednisone or its active metabolites prednisolone (PRED), or deflazacort (DFZ) (Wong and Christopher, 2002; Angelini et al., 1994; Biggar et al., 2001; Mendell et al., 1989). GC treatment is started optimally when patients are at their plateau of ability and before the decline phase (Hufton and Roper, 2017). Even if the therapy cannot heal the disease, it has been shown to delay LoA of average 3 years (Bello et al., 2015b), to preserve pulmonary function and to delay DCM and scoliosis onset (Lamb et al., 2016).

The importance of the environmental effect had been confirmed by a recent study by Hufton and Roper. Their research takes in consideration patients' socio-economic condition and shows that patients from South Asian and deprived backgrounds have earlier LoA because social and cultural factors influence access to treatment (Hufton and Roper, 2017).

### *"Cis" effect – DMD mutations*

The most common changes in dystrophin are deletions, which account for 65% of *DMD* mutations, followed by duplications (5-15%). These deletions are located almost anywhere in the dystrophin gene, however, there are two known deletion hotspots: one located towards the central part of the

gene and the other towards the 5' end. The first one is the most commonly mutated region and includes exons 45–55, while the 5' end hotspot includes exons 2–19 (Muntoni et al., 2003).

The "reading frame hypothesis" (Koenig et al., 1989) is the only rule to correlate mutations with disease severity in DMD. Nevertheless, several studies showed that not all mutations in *DMD* are equivalent (Bello et al., 2016b; Pane et al., 2014b; Wang et al., 2018).

Wang and colleagues analysed patients amenable to targeted skipping of exons 8, 44, 45, 50, 51, 52, 53, or 55, as well as exonic duplications or nonsense mutations. They demonstrated that patients amenable to exon 51 skipping are associated with earlier LoA compare to all other mutations (Wang et al., 2018). At the same time, patients amenable to exon 8 or exon 44 skipping manifest a milder phenotype compared to patients carrying all the other mutations (Bello et al., 2016b; Pane et al., 2014b; Wang et al., 2018) (Figure 5). Wang's findings are consistent with a model in which endogenous exon skipping in *DMD* transcripts results in a low level of in-frame mRNA and production of low levels of rescued dystrophin protein, which contributes toward reduction in disease severity as measured by delay in age of LoA in exon 44 or 8 skippable patients (Wang et al., 2018).

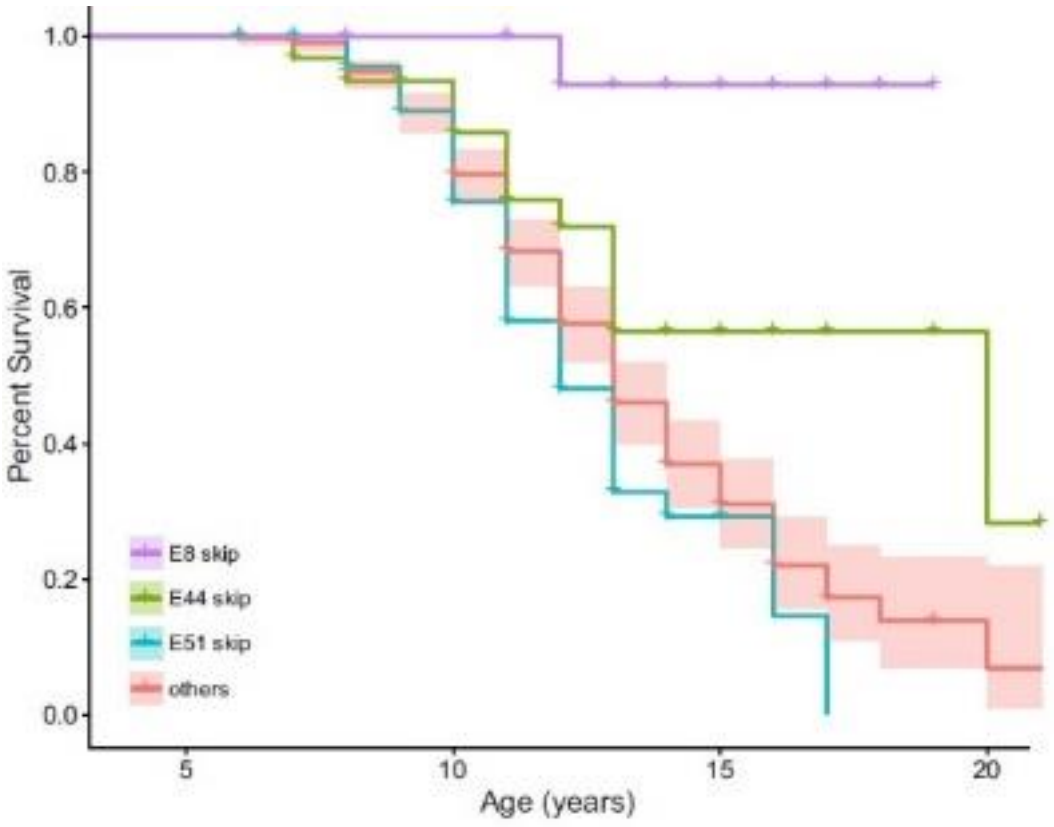


*Figure 5. Kaplan–Meier age at LoA analysis for patients eligible for skipping therapy of exons. Delayed age at LoA was observed among individuals amenable to exon 8 skipping (P<0.001) and exon 44 skipping (p-value = 0.04). Exon 51 skippable individuals had earlier age at LoA (p-value = 0.04). All other groups (45, 50, 52,53, duplication and nonsense) were not significantly different and were merged. All subjects were currently using corticosteroids* (Wang et al., 2018).

This idea was demonstrated also by Dwianingsih and colleagues for a patient amenable for exon 44 skipping. The patient, who had an out-of-frame deletion of exon 45, lost ambulatory capacities at 18-

year-old (later that the average for DMD that is age of 12). Despite the out-of-frame mutation, the muscle biopsy of the patient showed weak dystrophin staining. This finding was justified by the identification of partial exon 44 skipping, that leads to the production of Δ44/45 dystrophin mRNA. This transcript codified for a internally truncated protein, which was probably sufficient to elongate the independent ambulation period to 18 years (Dwianingsih et al., 2014).

It was estimated that possessing a *DMD* mutation that is amenable to exon 44 skipping is associated to a hazard ratio (HR) of 0.54 (Wang et al., 2018)/0.34 (Bello et al., 2016b). The data indicates that the impact of this mutation on the phenotype is roughly comparable with corticosteroid treatment (i.e., HRs for DFZ and PRED are 0.31 vs 0.62 (Wang et al., 2018) - 0.22 vs. 0.34 (Bello et al., 2016b)).

### *"Trans" effect – genetic background*

In addition to the standard of care and the effect of the *DMD* mutation, the variability observed in DMD patients phenotype can be due to the genetic background, in particular to genetic modifiers (Bello and Pegoraro, 2019). These are polymorphisms in genes - others than the one that causes the disease, which influence disease phenotype.

To identify genetic modifiers involved in Mendelian disease it is possible to follow hypothesis-driven or hypothesis-free approaches. The hypothesis-driven strategy involves association studies on candidate genes, on the contrary hypothesis-free method is based on genome-wide association studies (GWAS).

The first approach allows researchers to concentrate on selected variants in genes with a known role in the pathogenesis of the disease, established in previous preclinical and tissue-based studies. The focus in this strategy is on a scientific question that can be answered with good statistical power using relatively small samples.

On the contrary, the GWAS approach may allow the identification of new genetic modifiers. The downside related to this strategy is that it requires big sample size, that is very challenging to collect in rare diseases such as DMD, to reach the statistical significance (Bello and Pegoraro, 2019).

Currently there are 10 single nucleotide polymorphisms (SNPs) known to be DMD genetic modifiers, discovered using both hypothesis-driven and hypothesis-free approaches. They are *SPP1* rs28357094 (Pegoraro et al., 2011), *LTBP4* rs10880, rs2303729 and rs1131620 (Flanigan et al., 2013), *CD40* rs1883832 (Bello et al., 2016a), *ACTN3* rs1815739 (Hogarth et al., 2017), *THBS1* rs2725797 and

rs2624259 (Weiss et al., 2018). All these genes are involved in key features of DMD pathogenesis such as inflammation, fibrosis, response to treatment and muscle function (Figure 6).

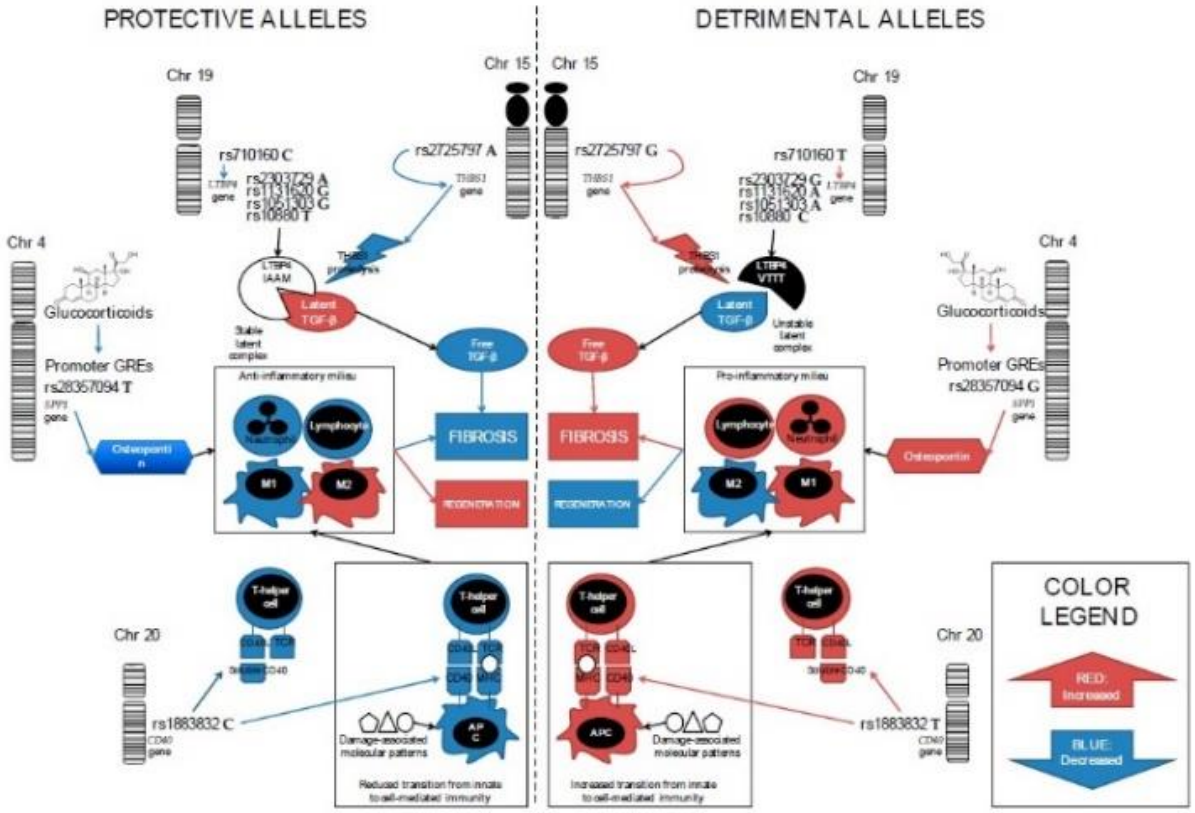


*Figure 6. schematic showing the molecular mechanisms proposed to explain the associations of modifier single nucleotide polymorphisms (SNPs) with Duchenne muscular dystrophy phenotypes. The colour blue indicates molecules, pathways, or biological processes that are reduced in association with a certain genotype; while the colour red indicates those that are increased. On the left side are represented protective alleles of the modifier SNPs, and their consequences; while the right side secularly represents detrimental alleles. Note that only genes with mechanisms related to fibrosis and inflammation are represented here. ACTN3, which modifies DMD through different mechanisms related to the sarcomere and muscle fibre type, is excluded (Bello and Pegoraro, 2019).*

### *SPP1* (Secreted PhosphoProtein 1, also known as Osteopontin)

rs28357094 in *SPP1* had been involved in modulation of DMD phenotype in 2011 by Pegoraro and colleagues (Pegoraro et al., 2011). To investigate DMD phenotype variability, researchers performed a gene expression array (GWAS approach) on skeletal muscle biopsies obtained from DMD patients showing severe (poor response to GC treatment and early LoA) or milder phenotype (excellent response to treatment and late LoA), looking for differentially expressed genes. The analysis led to the identification of several genes, and the literature was searched for single nucleotide polymorphisms (SNPs) with known functional effect within these genes.

Among the selected SNPs there was rs28357094 in *SPP1* - encoding osteopontin (OPN). *SPP1* resulted to be significantly overexpressed in muscle samples from patients with severe DMD. rs28357094, located 66 bp upstream the starting codon, was predicted to alter the gene's transcriptional activity. In particular, the SNP is located in correspondence to the putative binding site for the ubiquitous

transcription factors Sp1; it had been demonstrated that the G allele determine the reduced binding of Sp1, and subsequently reduced production of OPN (Giacopelli et al., 2004).

It was possible to estimate the effect of rs28357094 as approximately one-year earlier LoA in patients carrying at least one copy of the minor allele G (dominant model) (Bello et al., 2012; Bello et al., 2015a; Pegoraro et al., 2011). The minor allele frequency (MAF) of rs28357094 in populations of European ancestry is around 24%, meaning that a considerable proportion of DMD patients is at risk of earlier LoA because of this genotype (Pegoraro et al., 2011).

Osteopontin (OPN) is a cytokine that belongs to the family of small integrin-binding ligand N-linked glycoprotein secreted phosphoproteins (Many et al., 2016). It was firstly identified as an adhesive component of bone tissue extracellular matrix (Mark et al., 1987), but it is expressed in a wide range of cells and transcribed in 5 different isoforms, and can be secreted as a soluble cytokine or as a component of the ECM, while an intracellular form of the protein has been described in murine cells (Gimba and Tilli, 2013). OPN is extensively modified by glycosylation, phosphorylation, sulphation (Gimba and Tilli, 2013), and proteolytic cleavages by thrombin and matrix metalloproteinases (Yokosaki et al., 2002). The protein is involved in several biological processes including tissue repair, cancer, inflammation and fibrosis.

OPN role in skeletal muscle is various and shows differences between human and mouse. In contrast with human, reduced transcription level of *Spp1* (*SPP1* orthologous in mouse) had been observed in mice injured muscle (Hirata et al., 2003). Studies in mice had also suggested a regulatory role of OPN of macrophages invasion and the remodelling of damaged muscle tissue (Hirata et al., 2003).

Osteopontin had also been studied in DMD mice (*mdx*), specifically *mdx* were breaded with osteopontin knock-out mice generating the double-mutant strain (*dmm*), that mimics the genotype of DMD patients with at least one G allele in rs28357094. *dmm* mice showed less fibrosis, increased strength, and reduced amounts of TGFβ - which is a well-known driver of fibrosis in the later phases of DMD pathology (check Pathogenesis for more details) (YW Chen et al., 2005), compare to *mdx* mice (Vetrone et al., 2009). Furthermore, a shift from a pro-inflammatory M1 macrophages phenotype, to a pro-regenerative M2 macrophage phenotype in the *dmm* model had been demonstrated (Capote et al., 2016). These modifications of the inflammatory cell pool may explain the increased the efficiency of regeneration, and delay end-stage fibrosis (Capote et al., 2016). OPN role in regeneration is confirmed by myoblasts which secrete the protein during regeneration (Uaesoontrachoon et al., 2008).

In summary, DMD patients with at least one G allele in rs28357094 show more severe phenotype compare to who is carrying the TT genotype, on the contrary in *dmm* mice it is possible to observe a milder phenotype compare to *mdx* ones. These contrasting results are explained by Barfield and

colleagues who reported the presence of several enhancers elements upstream *SPP1* promoter. In particular, in DMD pathogenesis may be involved the NF-κB binding site - which signalling is known to be increased in DMD patients (YW Chen et al., 2005), and the glucocorticoids responsive element (GRE). Both these enhancers had be shown to increase OPN production only when the G allele in rs28357094 is present (Figure 7) (Barfield et al., 2014).

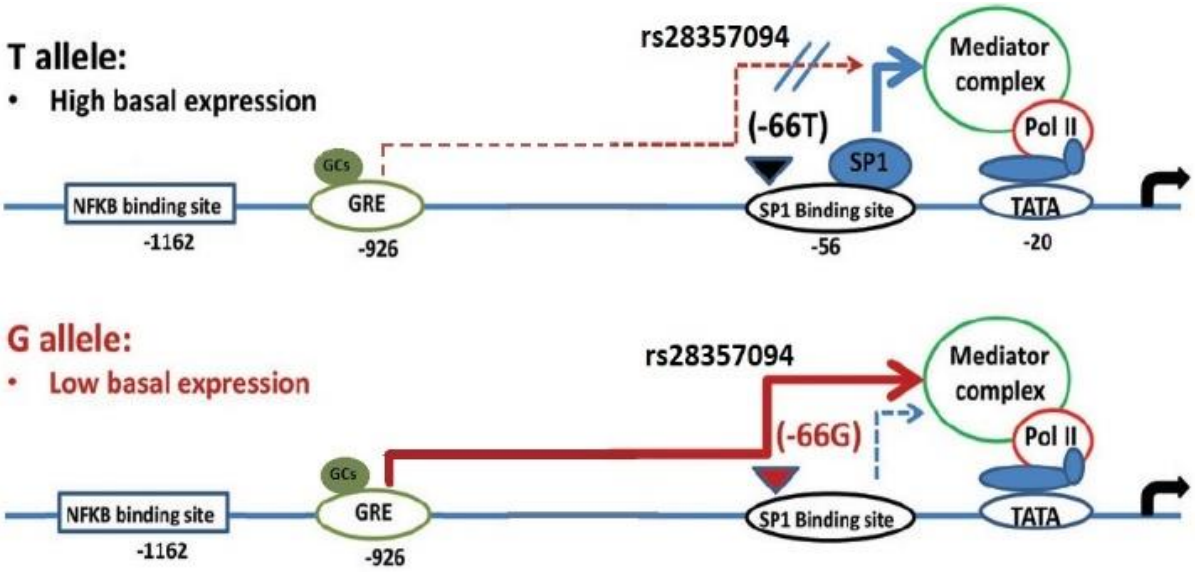


*Figure 7. Schematic of the OPN gene promoter structure, and proposed model for effect of genotype on OPN gene expression. The top two lines show the OPN gene promoter structure and proposed model for the allele-specific effects of the rs28357094 polymorphism on transcriptional regulation of SPP1. The predicted enhancer elements are in the more distal regions of the OPN gene promoter (nuclear factor κB binding site (NF-κB), GRE, glucocorticoid response element). Adapted from (Barfield et al., 2014).*

All together this suggests that DMD patients carrying the G allele may show greater NF-κB and/or glucocorticoid-induced transcription of *SPP1* during chronic inflammation, leading to exacerbation of the pro-inflammatory state of muscle and worsening of phenotype, as we have previously reported (Barfield et al., 2014). Further investigations on GCs effect on osteopontin expression are detailed in Aim 1 – *SPP1* genotype and glucocorticoid treatment modify osteopontin expression in Duchenne muscular dystrophy cells (Vianello et al., 2017)

Thanks to Barfield findings, recent studies, carried out to observe rs28357094 effect on DMD patients, were performed in stratified sub-populations, according to whether participants had been exposed to at least one-year of GCs treatment before LoA or last follow-up. This approach allowed to observe that GCs-treated subpopulation exhibited a larger rs28357094 effect, with two-year earlier LoA in patients with the dominant G genotype, while, in the GCs-untreated subpopulation, the median age at LoA was identical between the genotypes. This observation suggested the possibility of an interaction between *SPP1* genotype and glucocorticoids, so that the *SPP1* genotype becomes more relevant in GC-treated than untreated DMD populations (Bello and Pegoraro, 2019).

In summary, *SPP1* acts on both the "acute inflammation" features following early muscle damage in dystrophinopathy, and the efficiency of the regenerative process that follows. The acute inflammatory effect is probably mediated by the chemotaxis of neutrophils and lymphocytes, while the chronic pro-fibrotic effect by modulating myoblast proliferation and macrophage polarization. All of this happens under the complex influences of endogenous steroid hormones and exogenous therapeutic corticosteroids, so that *SPP1* genotype may be considered as a pharmacodynamic biomarker of the GC treatment response in DMD (Bello and Pegoraro, 2019).

### *LTBP4* (Latent Transforming Growth Factor β Binding Protein 4)

*LTBP4*, as well as *SPP1*, had been recognised as genetic modifier in DMD through a GWAS approach (genetic linkage study in mouse), followed by a candidate gene analysis (Heydemann et al., 2010).

Based on the idea that there is a shared pathological mechanism between DMD and diseases caused by loss of DGC proteins, Heydemann and colleagues generate γ-sarcoglycan-null mice interbreeding γ-sarcoglycan-null mice with different genetic backgrounds. The progeny showed dystrophic phenotype with different degrees of severity, which was well documented with data of muscle fibrosis and membrane permeability (Heydemann et al., 2010).

A genome-wide linkage study led to the identification of *Ltbp4* locus as the cause of the variability. Specifically, they observed both an insertion ($Ltbp4^{+36}$) and a deletion ($Ltbp4^{\Delta 36}$) in the same proline-rich region.

The human homologous of *Ltbp4* is *LTBP4*, that codifies for LTBP4 (Latent Transforming Growth Factor β Binding Protein 4) protein. It is preferentially expressed in cardiac, smooth and skeletal muscle upon myoblast differentiation and during regeneration (Giltay et al., 1997). It is co-secreted with TGFβ as LTBP4-TGFβ latent complex that releases TGFβ following proteolysis (Figure 8) (Chen et al., 2005; Sterner-Kock et al., 2002).

Both the polymorphisms found in Heydemann's mice ($Ltbp4^{+36}$ and $Ltbp4^{\Delta 36}$) are associated with LTBP4 altered proteolytic susceptibility. Specifically, the insertion appeared to be minimally susceptible to cleavage; the opposite phenotype was showed by the deletion, which sensitivity to cleavage is translated in increased TGFβ release from the matrix and increased binding of the molecule to TGFβ receptor on cell surface, leading to an increased activation of canonical TGFβ pathway (Figure 8).

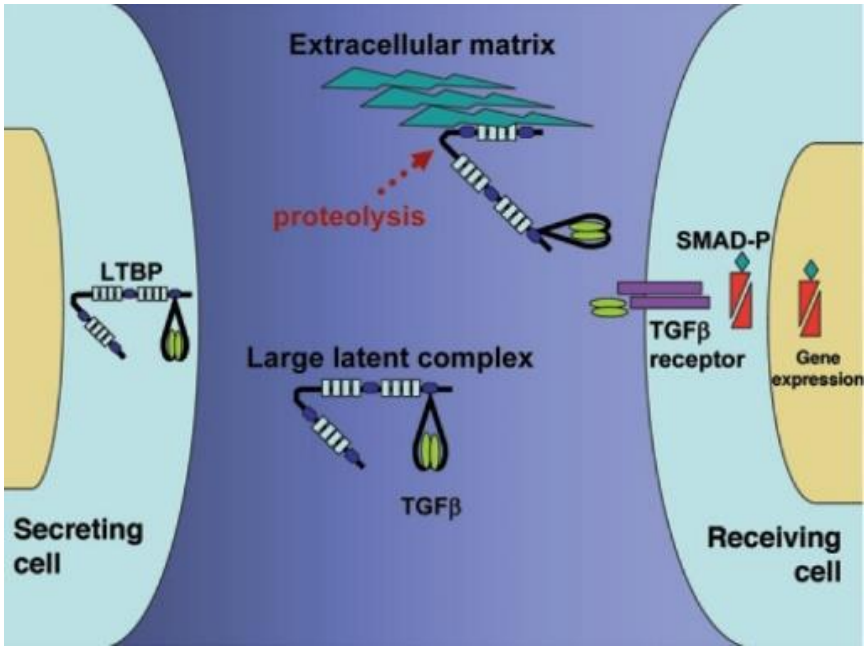


*Figure 8. Model of LTBP4 action. TGF-β forms the small latent complex with its inactive domain. The small latent complex binds to LTBP4 to form the large latent complex, where TGF-β is held inactive in the extracellular matrix. Proteolytic cleavage of LTBP4 releases TGF-β, it is now available to bind TGF-β receptors and activate the canonical TGF-β pathway (Heydemann et al., 2010).*

In human, in the same region carrying the polymorphisms in mice, there is a haplotype of 4 missense SNPs: rs2303729 (V194I), rs1131620 (T787A), rs1051303 (T820A) and rs10880 (T1140M), which are in moderate LD (linkage disequilibrium). It is possible to identify two common haplotypes (VTTT and IAAM) present in more than 80% of the population (Figure 9) (Flanigan et al., 2013).

The effect of the VTTT/IAAM haplotypes had been estimated in a cohort of patients from the United Dystrophinopathies Project (UDP), characterised by severe dystrophinopathy. The analysis established that IAAM delays of 1.5-2 LoA, with a recessive model. More specifically, all the SNPs of the IAAM haplotype are independently associated with delayed LoA, but the strongest effect is exerted when all the SNPs are present together (Flanigan et al., 2013).

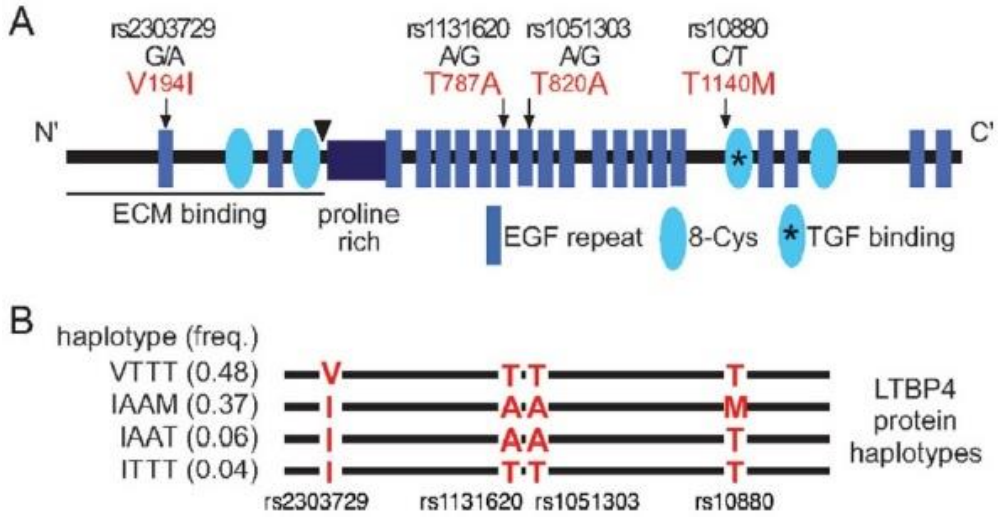


*Figure 9. LTBP4 protein and position of amino acid substitutions induced by SNPs. A) SNPs rs2303729, rs1131620, rs1051303, and rs10880 alter LTBP4; the amino acid substitutions are shown in red. B) LTBP4 protein haplotypes and frequencies estimated from 381 individuals found in the 1000 Genomes interim phase I data from populations with European ancestry (Flanigan et al., 2013).*

The effect of LTBP4 isoforms had been studied also *in vitro* in fibroblast with different genotypes of the modifier haplotypes (VTTT/VTTT, VTTT/IAAM, IAAM/IAAM). The study showed that in confluent conditions, with equal level of LTBP4 expression, the IAAM haplotype is associated with decreased TGFβ signalling. This is explained because IAAM haplotype codifies a LTBP4 isoform that cause increased sequestration of TGFβ because is more resistant to proteolytic cleavage and/or binds TGFβ more strongly. This may justify IAAM haplotype protective effect in DMD patients (Flanigan et al., 2013).

Further analysis showed that the effect of the VTTT/IAAM haplotype is not the same in all populations. In fact, VTTT/IAAM haplotype is in strong LD in population with European-American ancestry, but the LD is disrupted in African-Americans in whom minor haplotypes are more frequent (Bello et al., 2015a). This data suggests the involvement of other variants with different degrees of LD with the haplotype.

A second analysis of the UDP cohort confirmed this hypothesis, highlighting the presence of the SNP rs710160, located 12 kb upstream *LTBP4* promoter with a regulatory role on LTBP4 transcription activity. In particular, the minor allele C is associated with decreased in protein production (Weiss et al., 2018). rs710160 is unevenly distributed to VTTT/IAAM haplotype, phasing them it is possible to obtain three different haplotypes: T-VTTT (frequency= 0.49), T-IAAM (frequency= 0.13) and C-IAAM (frequency= 0.17) (Weiss et al., 2018). Among the 3, C-IAAM is associated with the milder phenotype. The hypothesis to explain this finding is that C-IAAM leads to low-expression of a highly proteolysis-resistant and strongly TGFβ-binding LTBP4 isoform, that cause the minor pro-fibrotic signalling and a milder phenotype among DMD patients (Weiss et al., 2018). These data are supported by further researches performed in *mdx* mice (Ceco et al., 2014; Lamar et al., 2016).

### *CD40 (Cluster of Differentiation 40)*, also known as *TNFRSF5* (Tumour Necrosis Factor Receptor SuperFamily Member 5)

Contrarily to *SPP1* and *LTBP4*, which had been discovered to be a genetic modifier with a mixed hypothesis-free and hypothesis-driven approach, *CD40* had been implicated in DMD phenotype modulation only through a hypothesis-free strategy. In particular, researchers performed an Exome Chip and then filtered the SNPs using a prioritising approach in which only SNPs within or 10 kb upstream/downstream gene involved in TGFβ and NF-κB pathways were considered (Bello et al., 2016a).

The analysis led to the identification of rs6074022 and rs4810485, two SNPs located around 6000 bp upstream the first intron of CD40. The validation of these SNPs was performed using a third SNP (rs1883832), located between the other two in perfect LD with them. rs1883832 T allele had been associated with earlier LoA, but its size effect varied among the cohorts used for the validation. Pooling all of them together the effect size was estimated around 1 year earlier LoA, the data was confirmed both using the dominant (p-value = 0.002) and the additive (p-value = 0.02) model (Bello et al., 2016a). *CD40* codifies TNRFSF5, a co-stimulatory protein expressed in both healthy and DMD muscles (Bello et al., 2016a). It is located on the surface of antigen presenting cells (APCs), involved in T helper polarisation.

The rs1883832 is adjacent to the translation start of CD40, the T allele had been shown to disrupt a relevant Kozak sequence (Jacobson et al., 2005), and the G allele in rs6074022 is associated with decreased transcription of *CD40* (Gandhi et al., 2010). The minor allele haplotype of these two polymorphisms had also been associated with an increased rate of alternative splicing of Δ-exon 6-secreted isoform of CD40. This isoform lacks the transmembrane domain and being secreted is predicted to interfere with CD40-CD40L signalling, preventing in this way cell-cell interaction between APCs and T-cells (Onouchi et al., 2012).

In conclusion, the mechanisms through which *CD40* modulates DMD phenotype appears to be complex and is not completely clear yet. What is known is that the haplotype of rs1883832 and rs6074022 has a complex effect at the transcriptional and translational level and involves tissue- and disease-specific mechanisms (Bello et al., 2016a).

### *ACTN3* (α-actinin 3)

α-actinins are major structural components of Z-lines, the structure that anchors actin filaments and maintain the spatial relationship between myofilaments (Blanchard et al., 1989). In humans, α-actinins are encoded by *ACTN2* (α-actinin-2) and *ACTN3* (α-actinin-3). The first isoform is express in all skeletal muscle fibres, whereas *ACTN3* expression is limited to type 2B (fast) fibres (Beggs et al., 1992; North

and Beggs, 1996). There is a common polymorphism in *ACTN3* (R577X, rs1815739) that determines the complete lack of protein production (North et al., 1999) and it is likely that α-actinin-2 is able to "compensate" for the absence of α-actinin-3 in type 2 fibres (Yang et al., 2003).

*ACTN3* had been demonstrated to be one of the factor that impacts on normal muscle function (Yang et al., 2003). In particular, it had been shown that sprint athletes have a lower frequency of XX and RX genotype and higher frequency of RR, compare to controls. On the other hand, endurance athletes have a slightly higher frequency of XX genotype than controls. It is important to notice that allele frequencies in sprint and endurance athletes go in opposite directions and differed significantly from each other (Yang et al., 2003). These findings suggest that the R allele provides an advantage for power and sprint activities. The reason is likely related to the fact that α-actinin-3 is the predominant fast fibre isoform in mouse and human (Mills et al., 2001) and may confer a greater capacity for the absorption or transmission of force at the Z-line during rapid contraction. Moreover, α-actinin-3 may promote the formation of fast-twitch fibres or alter glucose metabolism in response to training.

*ACTN3* effect on muscle performance in humans, can be also observed in *Actn3* knockout mice, which show reduced muscle mass and strength, with increased in endurance capacity, fatigue resistance and response to training (MacArthur et al., 2008). In these mice, fast 2B fibres, usually expressing α-actinin-3, display a shift towards a phenotype usually associated with slow fibres. The shift is likely due to a combination of metabolic and signalling changes caused by the absence of α-actinin-3. Specifically, it had been observed increased glycogen storage (Quinlan et al., 2010) and increased calcineurin activity (Hogarth et al., 2017).

The study of *ACTN3* polymorphism in human and the data from *Actn3* knockout mice, suggested that *ACTN3* may act as genetic modifier of DMD, and that the shift to towards slower muscle metabolic properties associated with α-actinin-3 lack may have a protective effect on DMD pathogenesis (Hogarth et al., 2017).

Hogarth and colleagues studied α-actinin-3 lack effects on *mdx* mice, generating a double mutant *Actn3*$^{-/-}$ *mdx*. These mice showed a significant decrease in force deficit following eccentric contraction compared with *mdx*, and were able to recover from fatigue considerably better than *mdx* (Hogarth et al., 2017).

In the same study, *ACTN3* effect as DMD genetic modifier had been proved also in humans. In particular, heterozygous patients shown reduction in strength and LoA in average 1-2 years before homozygous, with no differences observed between RR and XX. Since LoA is a complex phenotype, the grip strength was evaluated. Using this parameter as outcome, XX individuals showed a favourable gradient to RR, indicating that the α-actinin-3 deficiency may protect against the loss of strength over time as expected (Hogarth et al., 2017).

The singularity of heterozygous phenotype detected in LoA, may be because the loss of a single *ACTN3* allele does not drive muscle adaptation enough to ameliorate the progression of the dystrophic phenotype, and at the same time heterozygous presumably suffer the reduction in strength associated with α-actinin-3 haploinsufficiency. This hypothesis is supported by preliminary data obtained from *Actn3*$^{+/-}$ *mdx* mice (Hogarth et al., 2017).

Finally, the interpretation of results obtained from human data is make more complicated by sample stratification, as the X allele is more frequent in non-European populations, who might also present a different disease severity because of standard of care disparity or different genetic background (Hogarth et al., 2017).

### *THBS1* (thrombospondin-1)

The same GWAS study that confirmed *LTBP4* as genetic modifier for DMD also found other two SNPs (rs2725797 and rs2624259) strongly associated with variation in LoA in DMD patients (recessive model p-value = $6.6 \times 10^{-9}$; additive model p-value = $7.5 \times 10^{-6}$), in particular the minor allele of rs2725797 is protective for LoA (Weiss et al., 2018). rs2725797 and rs2624259 are in close proximity and strong LD. They are in a gene desert (region devoid of protein-coding genes) about 750 kb telomeric to thrombospondin-1 (*THBS1*) gene. *THBS1* is highly expressed in paediatric skeletal muscle, it is a major activator of TGFβ signalling, it directly interacts with LTBP4 and ECM (Murphy-Ullrich and Poczatek, 2000); moreover thrombospondin-1 expression is elevated in *mdx* mice (Cohn et al., 2007). The authors found a long-range chromatin interaction between the region in LD with the SNPs and *THBS1* promoter, specifically a CCCTC-binding factor (CTCF) binding site which is known to mediate long-range interactions between distant enhancers and their promoters. The regulatory role of the SNPs region is supported also by the presence of a DNase I hypersensitive site, which is indicative of open chromatin and cis-regulatory element in the same region. Further analysis showed that rs2725797 minor allele, the same implicated in delayed LoA, is associated with reduced *THBS1* expression in skeletal muscle (Weiss et al., 2018).

# Aims

## Aim 1

*In vitro* study of the genetic modifier *SPP1* (rs28357094) in DMD and control myotubes and myoblasts, with a focus on the effect of deflazacort treatment.

## Aim 2

Evaluation of the effect of glucocorticoids treatment, *DMD* mutations and modifier SNPs on DMD phenotype variability. For this purpose, we used several disease aspects (i.e. performance of upper limbs, respiratory and cardiac function) as outcome values in our analyses.

## Side project

Modelling of Duchenne muscular dystrophy and Central core disease in a three-dimensional *in vitro* system, to shine a light on diseases' pathophysiological aspects and to provide an effective platform to drug screening and testing.

# Aim 1 – *SPP1* genotype and glucocorticoid treatment modify osteopontin expression in Duchenne muscular dystrophy cells (Vianello et al., 2017)

As previously described, the SNP rs28357094 in *SPP1* is a Duchenne muscular dystrophy genetic modifier (refer to "*SPP1* (Secreted PhosphoProtein 1, also known as Osteopontin)" for more details). It had been shown that the modulatory effect of the polymorphism is due to the interaction with glucocorticoids, which are the main treatment for DMD patients (Barfield et al., 2014).

When I joint Professor Pegoraro's lab, I took part in an ongoing research that aimed to further elucidate the role of rs28357094 genotype on osteopontin expression. We investigated OPN expression in myoblasts and myotubes obtained from DMD patients and healthy controls - with defined rs28357094 genotypes, muscle biopsies. 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 (Vianello et al., 2017).

## Material 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*

11 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. 9 normal paediatric muscle biopsies were used as controls (mean age at biopsy ± standard deviation 6.8 ± 5.1 years). All muscle biopsies from patients 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 min. After digestion, muscle fragments were gently dissociated by pipetting with a 2 ml

and 1ml pipette, before being passed through a 21G 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 35mm well in growth medium composed of 20% FBS, 25 ng/ml 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 100mm dishes.

### *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, 134mM 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. CD56þcells 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 1% 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 35mm wells. Proliferating myoblasts or differentiating myotubes were treated with vehicle (untreated cells), 0.1 $\mu M$ or 1 $\mu M$ 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, 1mg 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.

### *Western blot*

Proliferating myoblasts and differentiated myotubes were washed twice in PBS, and incubated in 100 ml of 10mM Tris (pH 6.8), 1mM EDTA, proteinase inhibitor cocktail (Roche; Basel, Switzerland) for 15 min on ice. 10 ml 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 mg of protein per sample were loaded onto 10% or 12% glycine-tris large-sized acrylamide gels. Proteins were blotted onto a 0.45 lm nitrocellulose membrane (GE Healthcare; Waukesha, WI, USA) in transfer buffer. Membranes were saturated in 5% TBST milk at room temperature for 1h 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. 50nM of siRNA oligos and 15 ml of Lipofectamine in 1ml Optimem (Invitrogen; Carlsbad, CA, USA) were used for 35mm 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 50kDa Western blot band; OPN protein expression evaluated by densitometry of the 55kDa 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 (2). 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 found that several OPN isoforms are differentially expressed in different stages of muscle cell maturation. Through western blot analysis, it was possible to detect a 50 kDa OPN isoform in muscle biopsy samples, a 55 kDa isoform in primary myoblasts, and both the isoforms were detectable in primary myotubes; while recombinant human OPN was observed at a molecular weight (MW) between 55 and 50 kDa. No differences in isoforms expression had been detected between control and DMD samples (Figure 10 A).

The specificity of the observed band was confirmed performing a transient siRNA mediated *SPP1* silencing in differentiated myotubes, in which both 55 and 50 kDa bands were observed (Figure 10 B-D). 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 50 kDa and 55kDa bands. Validation experiments with two alternative anti-OPN antibodies confirmed an OPN band at 55 kDa.

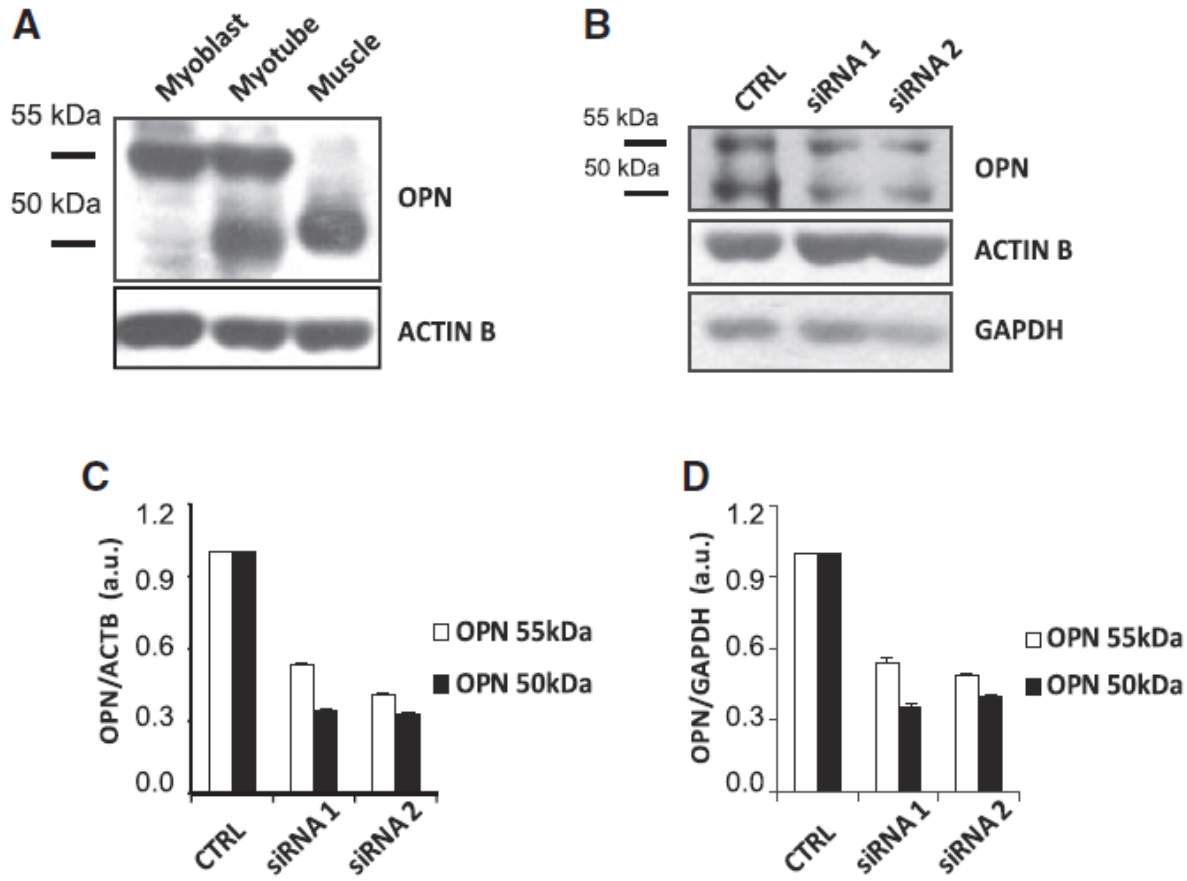


*Figure 10. OPN protein is present with different MW in human myoblasts, myotubes and muscle. A) Western blot of total protein lysates obtained from DMD myoblasts, myotubes and muscles showing a developmentally regulated OPN isoform expression. B) Western blot of total protein lysates obtained from control myotubes transfected with mock and two distinct siRNA oligos against human OPN. Down-regulation of both 55 and 50 kDa bands was quantified with densitometric image analysis (C, D).*

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

It is known that OPN expression is increased in DMD muscle biopsies (Piva et al., 2012). To confirm this data in myogenic cells, OPN and *SPP1* mRNA were quantified, with WB and RT-PCR experiments respectively, in myoblasts and myotubes isolated from 11 DMD patients and 9 age-matched normal controls. These analyses found no difference in *SPP1* expression, both at mRNA and protein level, between control and DMD myoblasts (Figure 11 A-D).

On the contrary, after differentiation, we found an increase of *SPP1* mRNA expression in DMD compare to control, even if the data is not statistically significant (paired Wilcoxon Test=0.053) (Figure 11 E). As expected from RT-PCR analysis, we also observed a significant increase in 50 kDa OPN in both DMD and control cultures (p-values = 0.021 and 0.015 respectively). The increase was more marked in DMD cultures, although the Mann-Whitney test comparing DMD to control myotubes was not significant (Figure 11 F). On the other hand, there were no significant changes in the expression of OPN evaluated as the 55kDa isoform or total OPN (Figure 11 G, H).

All 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 the 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 (Hoffman et al., 2013; Piva et al., 2012).

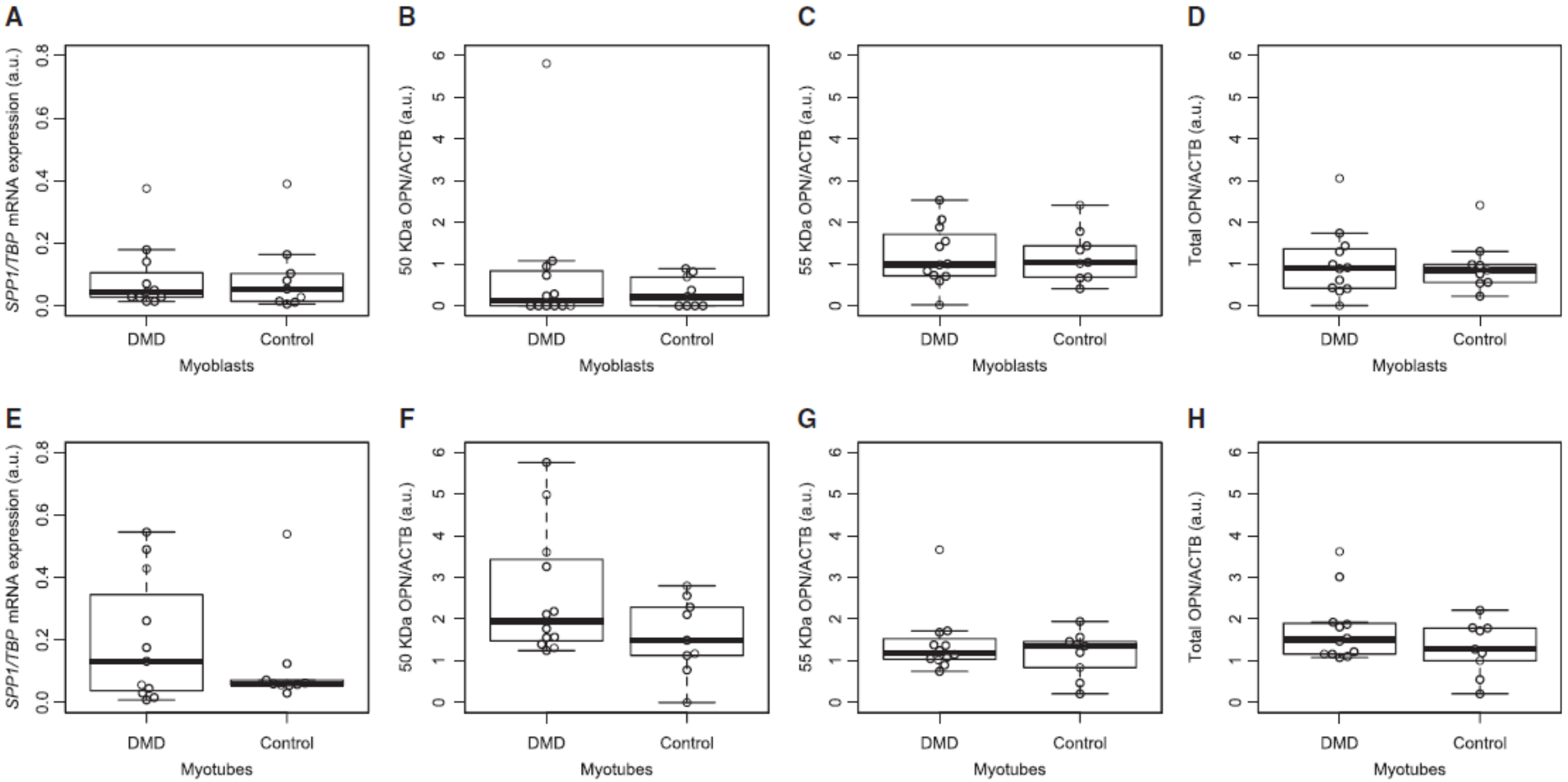


*Figure 11. SPP1 mRNA and OPN expression in DMD and control myoblast and myotube cultures. A–D) Summarize data from myoblast cultures, while (E–H) illustrate data from myotube cultures. A) and E) refer to SPP1 transcript expression levels assessed by RT-PCR; B) and F) refer to OPN expression evaluated 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

It is known that the SNP rs28357094 in *SPP1* promoter alters gene transcription (Giacopelli et al., 2004), to confirm this data in our primary lines, 11 DMD (7 TT, and 4 TG) and 9 age-matched healthy myogenic cell lines (5 TT, and 4 TG) were stratified based on their genotype.

WB and RT-PCR analyses carried in myoblasts, showed no significant differences between genotypes neither in *SPP1* mRNA expression (Figure 12 A) nor in 50 kDa OPN expression (Figure 12 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-value = 0.018 for 55 kDa, total OPN p-value = 0.003) (Figure 12 C,D). Considering only DMD population, the difference remains statistically significant for total OPN (p-value = 0.018, 55 kDa OPN p-value = 0.07).

Surprisingly, opposite results were obtained from myotubes, where the TT genotype shown an increase in 55kDa OPN and total OPN compare to the TG genotype (Mann-Whitney U test p-value = 0.018 for 55 kDa, and p-value = 0.003 for total) (Figure 12 G,H). These differences are significant also considering only DMD cultures only (p-value = 0.01).

Finally, no differenced were detected in *SPP1* mRNA or 50 kDa OPN expression between genotypes (Figure 12 E, F) (Vianello et al., 2017).

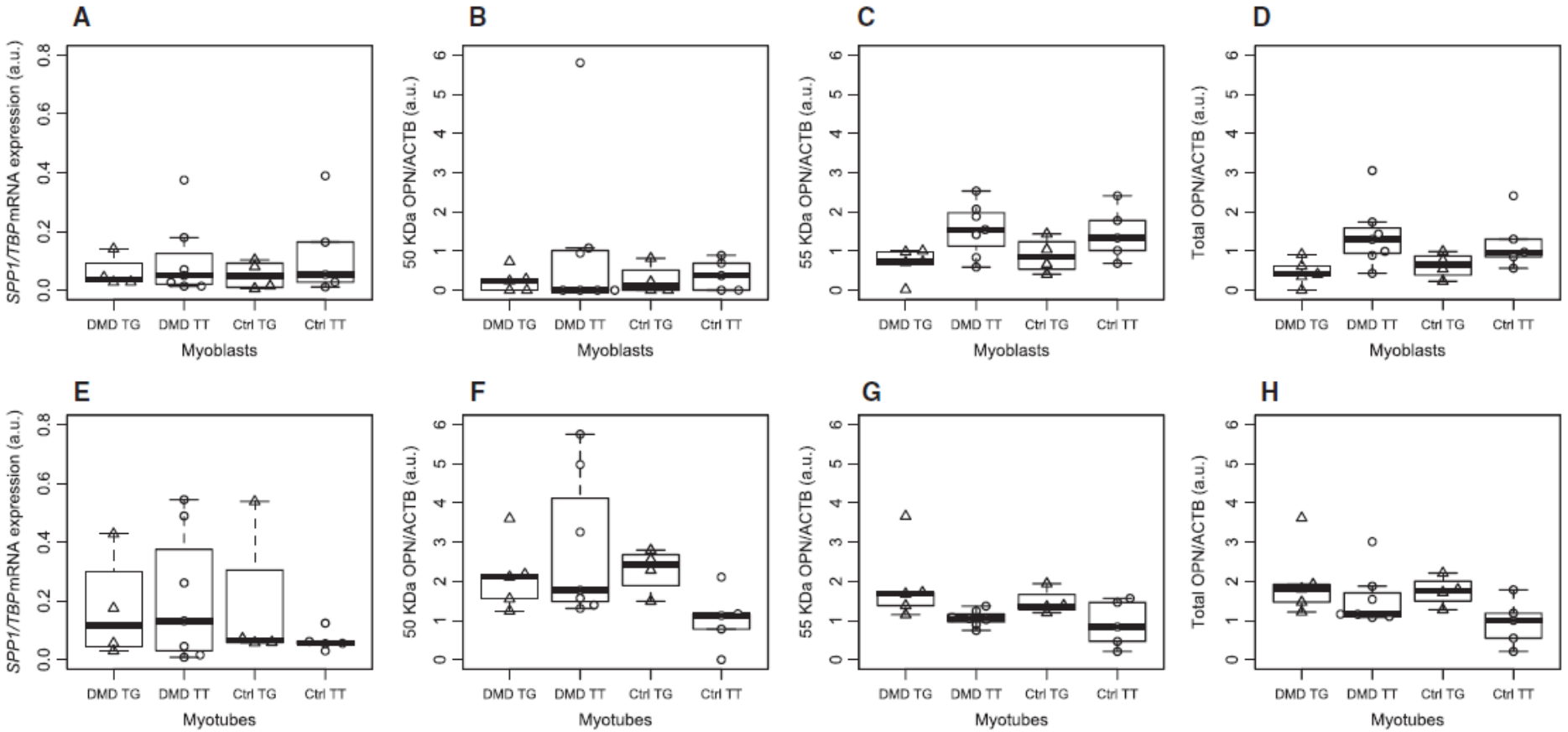


*Figure 12. SPP1 mRNA and OPN expression in DMD and control myoblast and myotube cultures, stratified by SPP1 rs28357094 genotype. A–D) summarize data from myoblast cultures, while E–H) illustrate data from myotubes. A) and E) refer to SPP1 transcript expression levels assessed by RT-PCR, B) and F) refer to OPN expression evaluated by Western Blot as the 50 kDa band, C) and G) refer to OPN expression evaluated by Western Blot as the 55 kDa band, and D) and H) refer to OPN expression evaluated by Western Blot as the two bands combined.*

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

As previously mentioned, GCs treatment is the only treatment available to ameliorate DMD symptoms; specifically, treatment with DFZ resulted to be well tolerated by patients (refer to "Environmental effect – standard of care" for more details).

We treated DMD and control cell lines with 0.1 µM and 1 µM DFZ, to test if the drug influences *SPP1* mRNA or OPN expression in DMD myoblasts and myotubes, and if this response is modified by *SPP1* rs28357094 genotype.

RT-PCR showed that DFZ treatment did not modify *SPP1* mRNA expression in control and DMD cultures from "TT" patients, while a non-significant increase in transcript quantity was noted with in DFZ-treated DMD cultures from "TG" patients (repeated measures ANOVA p-value = 0.058) (Figure 13 A).

Moreover, in myotubes, baseline levels of *SPP1* mRNA expression were slightly higher, especially in DMD and rs28357094 TG samples. 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 13 E).

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

DMD and control cultures treated with 0.1 µM and 1 µM DFZ were also analysed with WB. In myoblasts, the 50 kDa OPN band was detected at low levels at baseline, except in "TT" DMD cultures which showed higher levels. In this subgroup, the 50 kDa band intensity decreased slightly, although not significantly, with increasing concentration of DFZ (Figure 13 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-value = 0.012). The 55 kDa band of OPN and total OPN showed a slight decrease with increasing DFZ concentrations in all subgroups, but the statistical significance was reached only for control samples carrying the TT genotype for both 55 kDa and total OPN (repeated measures ANOVA p-value = 0.038 and 0.033 respectively) (Figure 13 C-D).

In myotubes, expression of the low MW band was higher, but we did not observe any significant effects of increasing DFZ concentrations on OPN protein quantity 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 13 F-H). With 1 µM DFZ treatment, total OPN was higher in DMD myotubes carrying the TG, rather than TT genotype (p-value = 0.047).

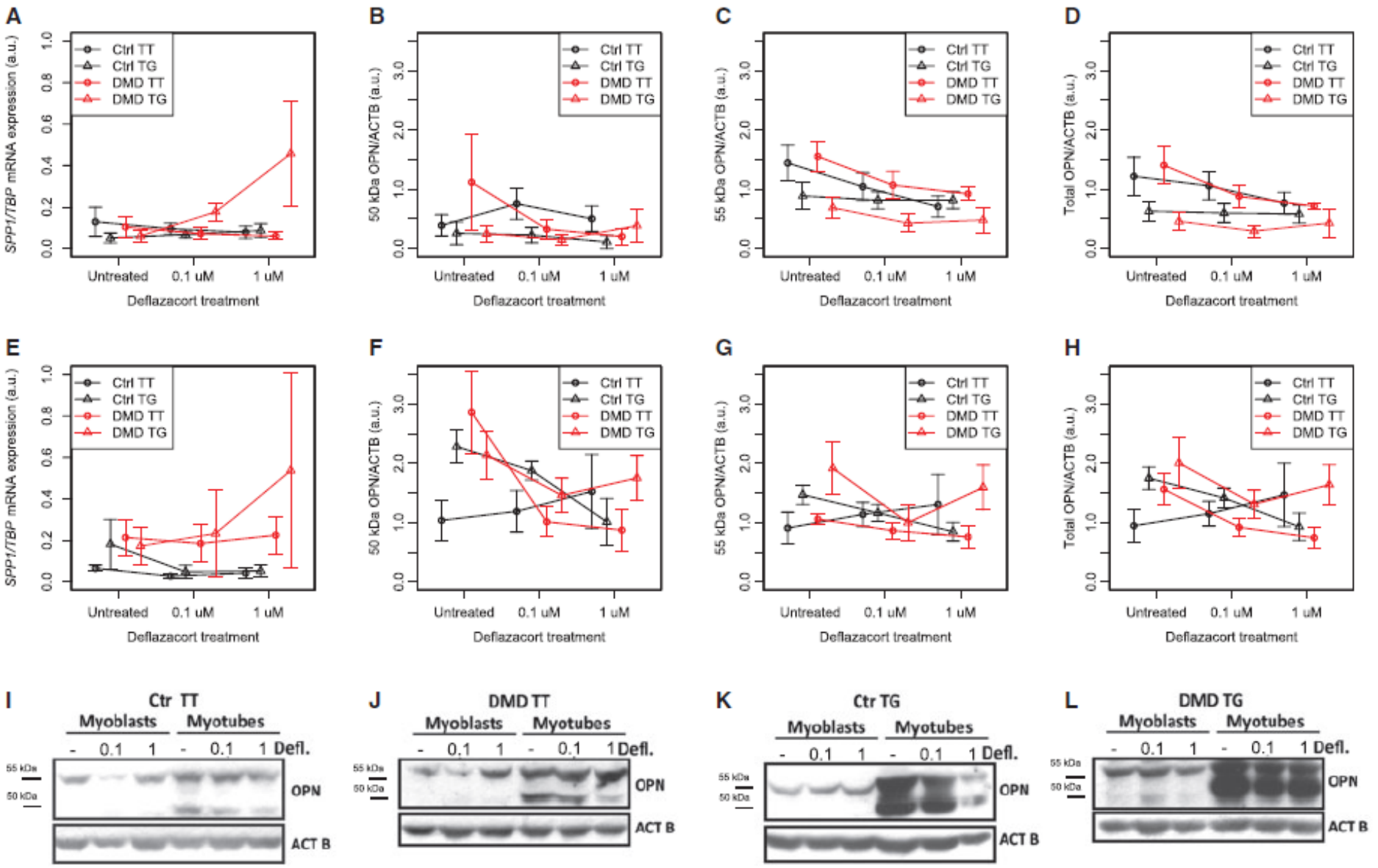


*Figure 13. OPN expression after treatment with increasing concentrations of deflazacort (Defl). SPP1 mRNA and OPN expression are represented as means (symbols) and their standard errors (bars). Lines connect data points for cultures from the same groups, at increasing deflazacort concentrations. A–D) refer to myoblasts 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').*

## 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 OPN 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 (Table 1). The ANCOVA analysis of *SPP1* mRNA expression in myoblasts showed significant interaction with rs28357094 genotype for both dystrophin deficiency and DFZ concentration (p-values = 0.029 and 0.020 respectively). This interaction is driven by the DFZ induced, dose-dependent mRNA increases in DMD "TG" samples (Figure 13 A). Again in myoblasts, the ANCOVA analysis of OPN band intensity showed strong significant independent effects of rs28357094 genotype on 55 kDa and total OPN (p-values = 0.001 and 0.0005 respectively), corresponding to the increased 55 kDa and total OPN band intensity in WB from "TT" cultures at baseline (Figure 12 C, 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-value = 0.029) (Figure 12 E), while the independent effects of rs28357094 on 55 kDa and total OPN protein quantity were barely significant (p-values = 0.050 and 0.048 respectively), although these effects were in the opposite direction as observed in myotubes (Figure 12, 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-values= 0.033 and 0.041, respectively).

*Table 1. Analysis of covariance (ANCOVA) of SPP1 mRNA and OPN protein expression: p-values for factors (lack of dystrophin, rs28357094 genotype, and DFZ concentration) and their interactions.*

| | | **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** | ***DMD* lack** | 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. | **0.05** | **0.048** |
| **Interactions** | ***DMD* lack * [DFZ]** | **0.085** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | ***DMD lack* * rs28357094** | **0.029** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **[DFZ] * rs28357094** | **0.02** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | ***DMD lack* * [DFZ] *rs28357094** | **0.073** | n.s. | n.s. | n.s. | n.s. | **0.033** | n.s. | **0.041** |

## Discussion

The role of genetic modifiers of DMD is becoming increasingly interesting given the potentially relevant implications they have for the 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 techniques has allowed genome-wide study of genetic variations in large populations in a cost-effective and highly efficient way (Bello et al., 2016a). 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 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 osteopontin 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 55kDa band, in differentiated myotubes as both a 55 and a 50kDa band, and in mature muscle as a single 50kDa 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 percentages 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., 2013). 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 and Tilli, 2013; Saitoh et al., 1995), and this also applies to skeletal muscle (Many et al., 2016). The molecular weights reported for splicing isoform OPN-a and OPN-b (54 kDa and 50 kDa, respectively) (Many et al., 2016) are close to those of the observed bands, and unpublished 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. 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 osteopontin observed in DMD muscle tissue compared to controls (Haslett et al., 2002; Piva et al., 2012; 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 (Pagel et al., 2014; Uaesoontrachoon et al., 2013). 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 physicochemical 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 fibres, resulting in muscle fibre 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 (Chen et al., 2005b; Petrof et al., 1993). Contraction-induced sarcolemma rupture triggers a variety of molecular changes including release of mitogenic factors (Allen et al., 1995; Chen et al., 1994; Tatsumi et al., 1998), cytokines (Bakay et al., 2002), and reactive inflammatory signature molecules (Porter et al., 2002; Uaesoontrachoon et al., 2008) that modify the muscle microenvironment. Since osteopontin is induced by skeletal muscle injury (Barbosa-Souza et al., 2011; Hirata et al., 2003; Hoffman et al., 2013; Kuraoka et al., 2016; Uaesoontrachoon et al., 2013), 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 (Uaesoontrachoon et al., 2008; Vetrone et al., 2009), 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 (Barfield et al., 2014; Giacopelli et al., 2004) 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 (Bello et al., 2015a; Pegoraro et al., 2011), 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 (Barfield et al., 2014; Giacopelli et al., 2004) 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 (Gloss et al., 2016; Matthews 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., 2015a; van den Bergen et al., 2015), and it has been proposed that osteopontin may be envisaged as a pharmacodynamic modifier of glucocorticoid response in DMD (Bello et al., 2015a; Pegoraro et al., 2011). Among steroids, deflazacort is emerging as potentially more effective (Bello et al., 2015a) 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 (Barfield et al., 2014; Hoffman et al., 2013). 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 led 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 (Hoffman et al., 2013). 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 osteopontin overexpression. In this scenario, dysregulated *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

We also acknowledge support from the Telethon Genetic BioBank (GTB12001D) and the Eurobiobank network.
Conflict of Interest statement. EP reports personal fees from Genzyme and PTC Pharmaceuticals, outside the submitted work.

## Funding

University of Padova (Grant CPDA151054) and the Telethon Genetic BioBank grant GTB12001D from Telethon Italia Foundation.

# Aim 2 - Genetic modifiers of performance of the upper limbs in Duchenne muscular dystrophy patients

## Introduction

The increasing number of clinical trials in Duchenne muscular dystrophy has highlighted the lack of reliable outcome measure in non-ambulant DMD. This resulted in trials manly targeting young ambulant patients, drastically restricting the population size, given the early loss of ambulation age in the affected boys.

To address the need of an outcome measure capable to evaluate ambulant and not-ambulant DMD patients, a group of clinicians, physiotherapists and representatives from advocacy groups and industries worked together in 2012 to develop the Performance of Upper Limb (PUL) test. This protocol is able to evaluate a wide range of upper limbs activities, from antigravity shoulder movements to finger movements, joining the advantages of observed-rated measures with ability to perform activities of everyday life (Mercuri et al., 2012).

With the present study we aim to evaluate the effect of known modifiers on upper limbs function in DMD, using PUL test scores as outcome measure.

For a better description of the natural history of the disease phenotype, we take in account in the study different types of *DMD* mutations, grouped by their amenability for exon-skipping therapies, with the aim to describe the PUL score trajectories for all the subgroups and provide useful information for clinical trial design.

## Methods

### *Cohorts*

We collected retrospective PUL data from several Italian Centres from February 2012 to November 2018, with the aim of describing the effects of GCS treatment, age, genetic modifiers and *DMD* mutations on upper limbs performance of DMD patients.

### *Ethics statement*

All participants or their parents/guardians provided informed consent to study procedures, which were carried out in accordance with the Declaration of Helsinki and approved by Ethics Committees/Institutional Review Boards at participating Institutions.

### *Inclusion criteria*

All patients included in this study present *DMD* mutations leading to absent or <3% dystrophin by immunohistochemistry (IHC, except revertant fibres) or immunoblot. All patients were also able to understand the instruction to correctly perform PUL items. Among the patients enrolled in the research, 12 have nonsense *DMD* mutation that allows the treatment with ataluren (Translarna), a drug that enables dystrophin production with a readthrough mechanisms. To eliminate source of variability due to potential disease-changing effects of ataluren, in a study focused on DMD natural history and GCs effects, PUL data obtained during the period in which patients had been treated with Translarna had been excluded from the analysis.

### *PUL test*

To evaluate upper limbs performance, we used PUL scale version 1.2 (see Appendix A). The test is composed of 22 items (from A to V), 21 of which assess the functionality of upper limbs, divided in 3 domains: proximal domain (henceforth "Shoulder") item B to E, medial domain (henceforth "Elbow") item F to N, and distal domain (henceforth "Distal") item O to V. Item A allows to evaluate overall proximal function; if the patient scores equal or less than 3, which means that he has lost the ability to raise his arms at the level of the shoulders, the test continues from item F. Total PUL score is calculated by the sum of all items, excluded item A.

### *DMD genotype*

Information about pathogenetic *DMD* mutations were collected when available from clinical records or genetic reports. We classified deletions based on amenability to molecular treatments, i.e. skipping of exons 8, 44, 45, 51, and 53 (henceforth: "skip 8", "skip 44", etc.). Nonsense and splice site mutations were also considered as separate groups.

The effect of different mutations amenable to molecular treatment on DMD natural history had been previously described. Brogna et colleagues found that skip 44, skip 45 and skip 53 mutations are modifiers of disease progression when longitudinal data about ambulatory abilities are considered as outcome (Brogna et al., 2019). For this reason, we evaluate mutations effects grouping them as "skip 44", "skip 45", "skip 53" and "other skips".

Moreover, all mutations were subdivided into "proximal", i.e. situated 5' of intron 44, and therefore not predicted to alter the expression of short dystrophin isoforms (Dp140, Dp116, and Dp71); and "distal", i.e. involving intron 44 and/or regions 3' of it, thus disrupting these isoforms. The hypothesis supporting this dichotomy is that "distal" mutations may be associated with central nervous system involvement that might secondarily affect gross and fine motor performance (Doorenweerd et al., 2017; Felisari et al., 2000; Magri et al., 2011).

### *Modifier genotypes*

Patients with available DNA samples were genotyped, using TaqMan (Thermo Fisher Scientific) assay, at all known DMD modifier loci: *SPP1* rs28357094 (Pegoraro et al., 2011), *LTBP4* rs10880, rs2303729 and rs1131620 (Flanigan et al., 2013), *CD40* rs1883832 (Bello et al., 2016a), *ACTN3* rs1815739 (Hogarth et al., 2017), *THBS1* rs2725797 and rs2624259 (Weiss et al., 2018). For tests of genotype/phenotype association, we used the same inheritance models as in published reports (Table 2). To exclude genotyping errors, alleles frequencies had been tested for Hardy-Weinberg equilibrium.

*Table 2. Genetic modifiers' published inheritance model.*

| **Genetic modifiers** | **Inheritance model** |
|---|---|
| *SPP1* rs28357094 | Dominant |
| *LTBP4* rs10880 | Recessive |
| *LTBP4 rs2303729* | Recessive |
| *LTBP4 rs1131620* | Recessive |
| *CD40* rs1883832 | Additive |
| *ACTN3* rs1815739 | Additive |
| *THBS1* rs2725797 | Recessive |
| *THBS1* rs2624259 | Recessive |

### *Statistical analysis*

Quantitative variables were summarized as mean ± standard deviation (SD) and median (range), unless otherwise specified. Intervals of linear decrease of PUL scores (total PUL, Shoulder, Elbow, and Distal) measures were defined on the age axis by piecewise regression, using baseline data (i.e. earliest available value) and choosing a 1-break model for total PUL, Elbow and Distal, and linear model without break point for Shoulder after visual inspection of the scatter plot. Generalized Estimating Equations (GEEs) were used to estimate effects of: age, GC treatment (on vs. off at each evaluation), *DMD* mutation (tested separately: each specified mutation group vs. "other" mutations; or "distal" vs. "proximal"), and SNP genotypes (dominant, recessive, or additive as appropriate).

In the GEE analysis covariates are considered to be independent one from the others, so only one of the SNPs of *LTBP4* haplotype had been included in the analysis (i.e. rs10880).

GEEs were applied within the "linear" age range defined by piecewise regression. Statistical significance was set at p-value <0.01 (Bonferroni correction for 5 genetic loci: *DMD*, *SPP1*, *LTBP4*, *CD40*, *ACTN3*). Statistical analyses were performed using R v.3.5.2.

# Results

## Demographics

We collected 670 result of PUL test from 146 DMD patients, specifically for 325 evaluations we were able to collect separately the score of the different domains, while for 345 measures only total PUL score was available. Patients underwent 3.79 ± 2.71 evaluations (maximum 13), with intervals of 0.59 ± 0.53 years, for a follow-up time of 2.69 ±1.69 years (maximum 6.19 years). Population average age at baseline was 11.16 ± 5.15 (maximum 28.57) years and at the end of the study it was 13.85 (maximum 32.42) years. The average LoA age is 11.46 ± 3.11 age.

## GC treatment

Glucocorticoids coverage of the studied population is summarized in Table 3.

*Table 3. Distribution by glucocorticoid treatment and demographics of treatment subgroups*

| Treatment subgroup | n (%) | Mean age in years ± SD | Median age in years (min - max) |
|---|---|---|---|
| Continuously off GCs | 35 (23.97%) | 16.41±6.90 | 16.33 (4.30 - 32.42) |
| Continuously on GCs | 87 (59.59%) | 12.07±4.68 | 11.35 (4.17 - 27.4) |
| Started GCs during FU | 4 (2.74%) | 9.76±2.59 | 9.895 (5.26 - 15.14) |
| Stopped GCs during FU | 11 (7.53%) | 15.96±4.75 | 9.5 (8.47 - 24.54) |
| Multiple switches | 1 (0.68%) | 5.46±1 | 5.46 (4.46 - 6.46) |
| Unknown or incomplete follow-up data | 8 (5.48%) | 14.86±5.6 | 13.2 (5.5 - 28.0) |
| **Total** | 146 | 11.16±5.15 | 10.08 (4.17 - 28.57) |

*GCs: glucocorticoids; FU: follow-up; n (%): population numerosity and percentage; SD: standard deviation; min-max: minimum and maximum age for each treatment subgroup.*

## *DMD* mutations

*DMD* mutations were defined in 64.38% of patients. As expected from literature (Flanigan et al., 2009; Juan-Mateu et al., 2015), single- and multi-exon deletions are the vast majority of observed defects in our cohort (66.96%), followed by nonsense mutations (10.71%) and duplications (9.82%) (Figure 14).

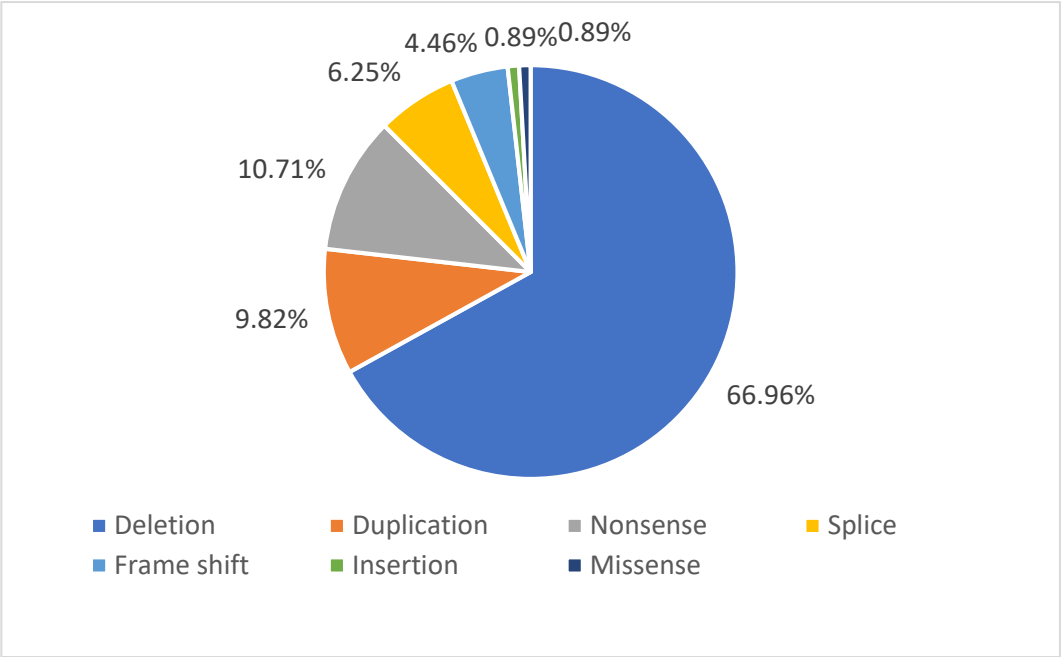


*Figure 14. Distribution of DMD mutation types.*

Mutations amenable for exon skipping therapy with known effect on ambulatory abilities are 15.18% and the group of other skips comprise of 9.82% of all mutations. Moreover, mutations were almost equally distributed among "proximal" (41.07%) and "distal" (58.92%) (Table 4).

*Table 4. DMD mutations distribution*

| **Mutation group** | | **n (%)** | |
|---|---|---|---|
| Deletions | skip 44 | 75 (66.96%) | 3 (2.68%) |
| | skip 45 | | 7 (6.25%) |
| | skip 53 | | 7 (6.25%) |
| | Other skips | | 11 (9.82%) |
| | Other | | 47 (41.96%) |
| Duplications | | 11 (9.82%) | |
| Nonsense mutations | | 12 (10.71%) | |
| Small FS mutations | | 5 (4.46%) | |
| Splice site mutations | | 7 (6.25%) | |
| **Total (molecularly defined)** | | 112 | |
| Proximal mutations (5' intron 44) | | 46 (41.07%) | |
| Distal mutations (3' intron 44) | | 66 (58.92%) | |
| **Total (molecularly defined)** | | 112 | |

*"skip 8": deletion amenable to treatment by antisense oligonucleotide promoting the skipping of exon 8; same for other exon numbers. FS: frameshifting.*

## Modifier SNPs genotype

It is well known that no genotyping method is completely free from errors, for this reason several methods have been developed to detect genotyping errors (Chen et al., 2017). In the present analysis we tested our data for deviation from Hardy-Weinberg equilibrium (HWE), which states that in a large,

randomly mating population, genotype frequencies should follow HWE equation ($p^2+2pq+q^2=1$). We found that HWE is respected for all the analysed loci, with the except of *THBS1* rs2725797, for which we observed less heterozygote than expected, for this reason rs2725797 has not been included in the statistical analysis (Table 5).

*Table 5. Genetic modifiers genotype*

| SNPs | Obs. major allele (p) frequency | Obs. minor allele (q) frequency | MAF in Europeans (not Finnish) | Obs. heterozygote (2pq) percentage | HWE heterozygote (2pq) percentage |
|---|---|---|---|---|---|
| ***SPP1*** **rs28357094** | 0.79 | 0.26 | 0.24 | 0.32 | 0.31 |
| ***LTBP4*** **rs10880** | 0.64 | 0.36 | 0.47 | 0.46 | 0.45 |
| ***ACTN3*** **rs1815739** | 0.54 | 0.42 | 0.44 | 0.54 | 0.53 |
| ***CD40*** **rs1883832** | 0.78 | 0.26 | 0.26 | 0.33 | 0.31 |
| ***THBS1*** **rs2725797** | 0.82 | 0.28 | 0.16 | 0.25* | 0.32 |

** data significantly different from expected (p<0.05). Obs.= observed; MAF= minor allele frequency; Hardy-Weinberg equilibrium (HWE): $p^2+2pq+q^2=1$. p = frequency of the dominant allele; q= frequency of the recessive allele; $p^2$ =frequency of individuals with the homozygous dominant genotype; 2pq = frequency of individuals with the heterozygous genotype; $q^2$= frequency of individuals with the homozygous recessive genotype.*

## Ranges of linear decrease

We used the piecewise regression model to estimate the ranges of linear decrease of total PUL and sub-domains scores. We found that PUL score decreases in a linear fashion starting from age of 7, particularly: total PUL breakpoint= 7.06 years (Figure 15 A), Distal breakpoint = 7.64 years (**Error! Reference source not found.** D), Elbow breakpoint = 8.66 years (Figure 15 C). Differently from other items, Shoulder score decreases linearly from baseline, so we used a linear model without breakpoint to study this domain (Figure 15 B).

## Effects of GCs and age

We used the generalised estimated equation (GEE) model to evaluate the effect of age and GCs treatment on PUL test scores, results are detailed in (Table 6). We estimated the rate of yearly decline (± standard error) of total PUL score as -2.70±0.22 of predicted. The effect entity of age seems to be variable on different items, but it always detrimental and highly significant (p-values <0.001).

With the same model we evaluated GCs treatment effect on PUL score. As highlighted in (Table 6), the protective role of the drug is confirmed in all items, even if it is not significant for proximal domain (p-value= 0.27). This data is also confirmed by the lower intercept values of untreated patients' regression lines compared to treated in the scatter plot of all items (Figure 15).

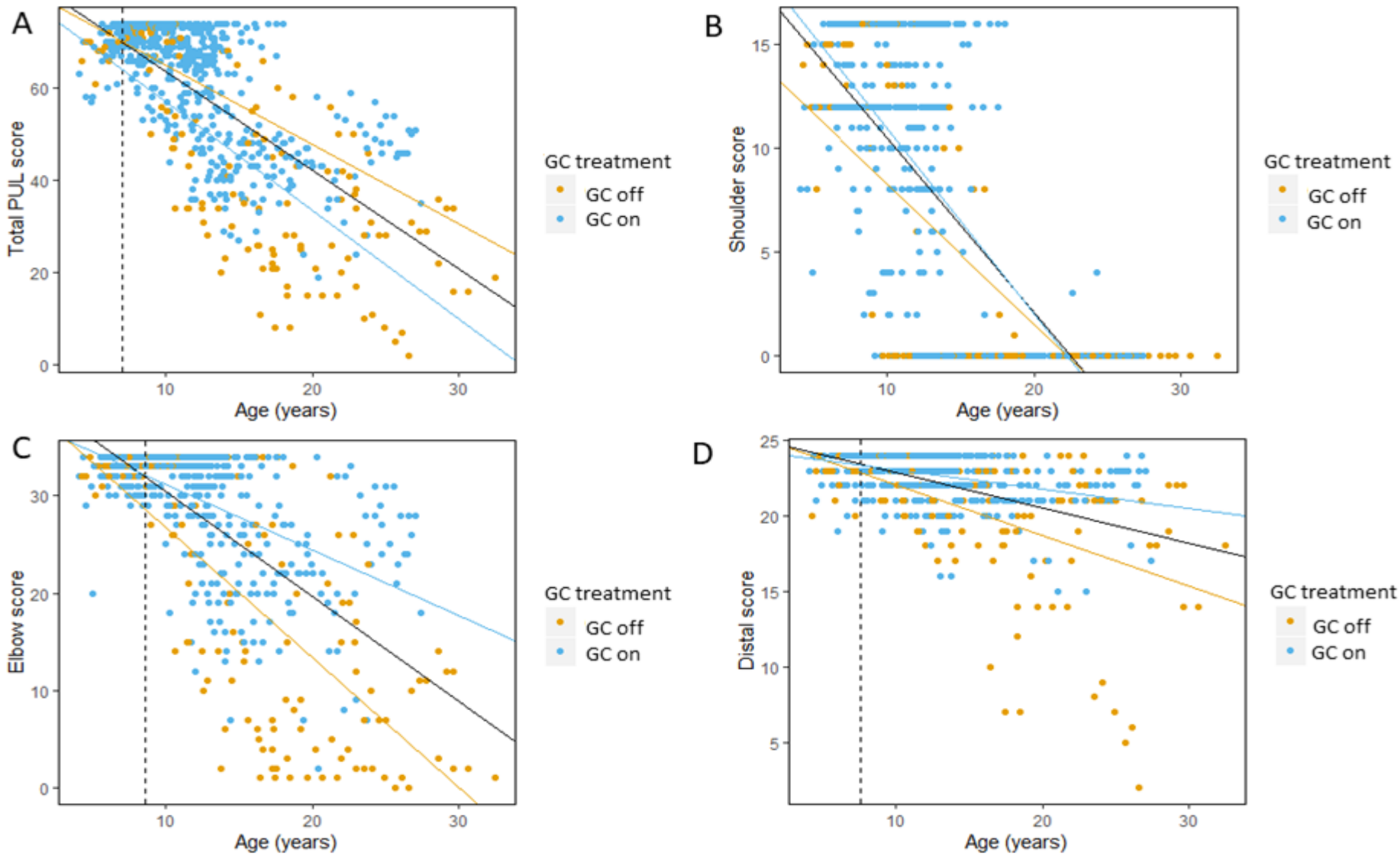


*Figure 15. Scatter plot of total PUL score (A), Shoulder score (B), Elbow score (C) and Distal score (D). The dashed vertical line indicates the limit of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model. Patients are grouped based on GCs treatment.*

### *DMD* mutation effects

With the methodology adopted in our study it was possible to confirm the detrimental effect of *DMD* mutations amenable to skip 53 and to identify these mutations as negative modifiers of DMD progression (Brogna et al., 2019). As reported in (Table 6), skip 53 is associated with lower scores in all the considered domains: total PUL score -12.67 ± 2.39; Shoulder score -3.14 ± 1.66; Elbow score -6.03 ± 1.76; Distal score -3.04 ± 1.58. The difference in highly significant (p-value < 0.001) for total PUL and Elbow score and nominally significant for Shoulder and Distal score (p-value = 0.028 and 0.03 respectively). Interestingly, we found that the group "other skips", in particular skip 8, has a significant (p-value <0.001) protective effect on Shoulder score (0.02 ± 1.49) (Figure 16 B).

Finally, we considered mutations based on their position on *DMD* gene, dividing them in proximal (5' intro 44) and distal (3' intron 44) and GEE model did not find significant differences linking mutations position and PUL score (Table 6).

### Modifier SNPs effects

None of the tested SNPs showed a significant effect on performance of upper limbs in our cohort after Bonferroni correction for multiple testing at 5 genetic loci (p-value <0.01). Nevertheless, *CD40* rs1883832 detrimental effect on total PUL score (-4.09 ± 1.90) and Shoulder score (-1.87 ± 0.79) resulted nominally significant (p-values = 0.03 and 0.02 respectively). Even if the effect of all other SNPs was not significant, we observed their effect on patients' performance mostly followed the expected trend (Figure 17-Figure 20) (Table 6).

*Table 6. Coefficients of Generalized Estimating Equation (GEE) analyses.*

| | | Total PUL score | | Shoulder score | | Elbow score | | Distal score | |
|---|---|---|---|---|---|---|---|---|---|
| **Coefficient** | | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** |
| **Intercept** | | 91.53 ± 3.9 | **< 0.0001** | 21.54 ± 1.93 | **< 0.0001** | 47.12 ± 2.86 | **< 0.0001** | 25.20 ± 0.86 | **< 0.0001** |
| **Age (per-year decrease)** | | -2.47 ± 0.22 ↓↓ | **< 0.0001** | -0.91± 0.11 ↓↓ | **< 0.0001** | -1.46± 0.15 ↓↓ | **< 0.0001** | -0.22± 0.04 ↓↓ | **< 0.0001** |
| **GC treatment** | | 5.00 ± 1.56 ↑↑ | **< 0.001** | 0.09 ± 1.00 | n.s. | 4.25 ± 0.9 ↑↑ | **< 0.0001** | 1.25 ± 0.56 ↑ | **0.025** |
| **Mutation 5' exon 44** | | 2.89 ± 2.28 | **n.s.** | 1.57 ± 0.97 | **n.s.** | 0.66 ± 1.40 | **n.s.** | 0.95 ± 0.40 | **n.s.** |
| **Mutation type** | **Other skips** | -4.86 ± 4.44 | n.s. | 0.02 ± 1.49 ↑ | **<0.001** | -3.77 ± 2.43 | n.s. | 1.14 ± 1.10 | n.s. |
| | **Skip 44** | 0.03 ± 9.56 | n.s. | 1.77 ± 2.73 | n.s. | 1.70 ± 9.01 | n.s. | -0.14 ± 1.12 | n.s. |
| | **Skip 45** | 2.48 ± 3.91 | n.s. | 0.97 ± 1.25 | n.s. | 1.63 ± 2.92 | n.s. | 0.86 ± 0.66 | n.s. |
| | **Skip 53** | -12.67± 2.39 ↓↓ | **< 0.0001** | -3.14± 1.66 ↓ | **0.026** | -6.03± 1.76 ↓↓ | **<0.001** | -3.40± 1.58 ↓ | **0.03** |
| **SNP modifiers** | **rs28357094 dom** | -2.40 ± 2.24 | n.s. | -0.78± 0.95 | n.s. | -2.24± 1.51 | n.s. | 0.16± 0.47 | n.s. |
| | **rs10880 rec** | -0.84 ± 2.60 | n.s. | -2.13 ± 1.23 | n.s. | 0.83 ± 1.66 | n.s. | 0.64 ± 0.75 | n.s. |
| | **rs1883832 add** | -4.09 ± 1.90 ↓ | **0.03** | -1.87 ± 0.79 ↓ | **0.02** | -1.77 ± 1.30 | n.s. | -0.63 ± 0.34 | n.s. |
| | **rs1815739 add** | 1.10 ± 1.41 | n.s. | 0.25 ± 0.66 | n.s. | -1.09 ± 0.90 | n.s. | -0.62 ± 0.39 | n.s. |

*SE: Standard Error; GC: glucocorticoid corticosteroids; Skip 8: mutations amenable to treatment with skipping of exon 8 (same for other exon numbers); n.s.= not significant; dom= dominant inheritance model; add= additive inheritance model; rec= recessive inheritance model. Nominally significant effects have been marked with arrows, upward for positive effects and downward for negative. Double arrows indicate "strong". p-values < 0.06 (nominally significant) or lower are highlighted in **bold**.*

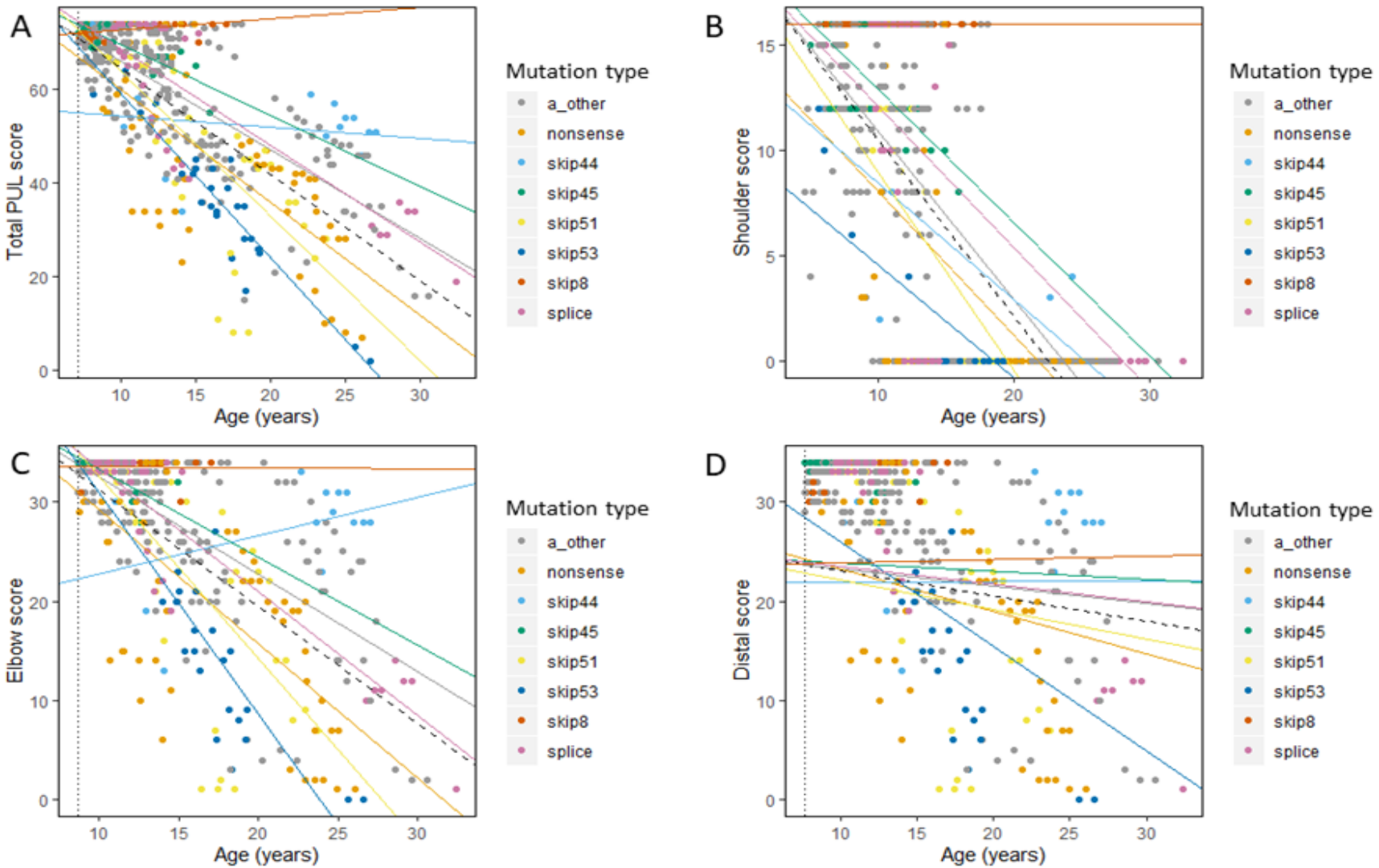


*Figure 16. Scatter plots of PUL scores (total PUL, A; Shoulder, B; Elbow, C; Distal, D) by age, grouped by DMD mutation type. Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model. The black dashed line is the regression line of the population, colourful solid lines are the regression lines of the mutation groups.*

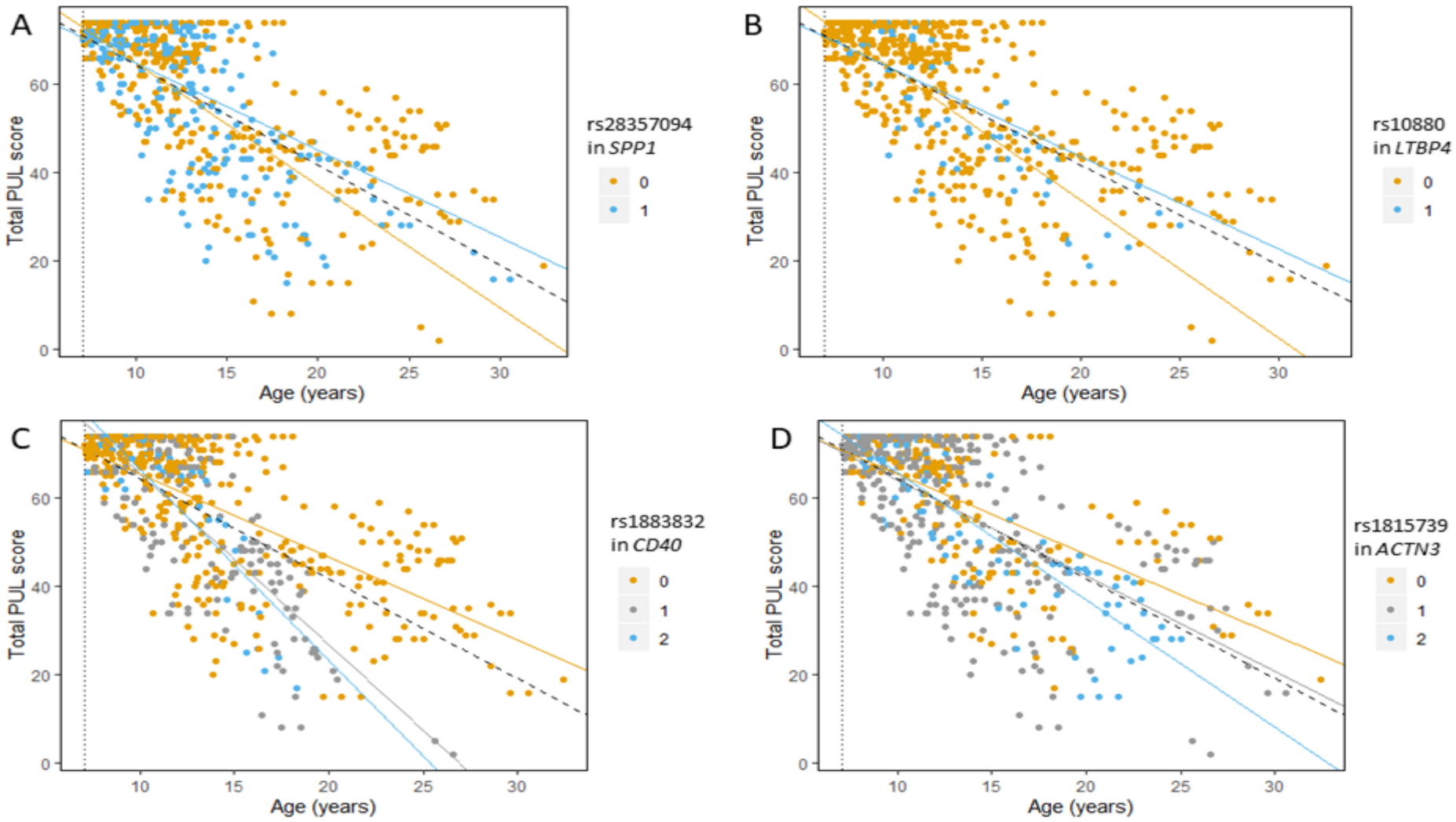


*Figure 17. Scatter plot of total PUL score by age. Data are grouped by modifiers SNPs genotype; alleles counting is shown based on inheritance model recommended in literature. Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model. The black regression lines describe the whole population.*

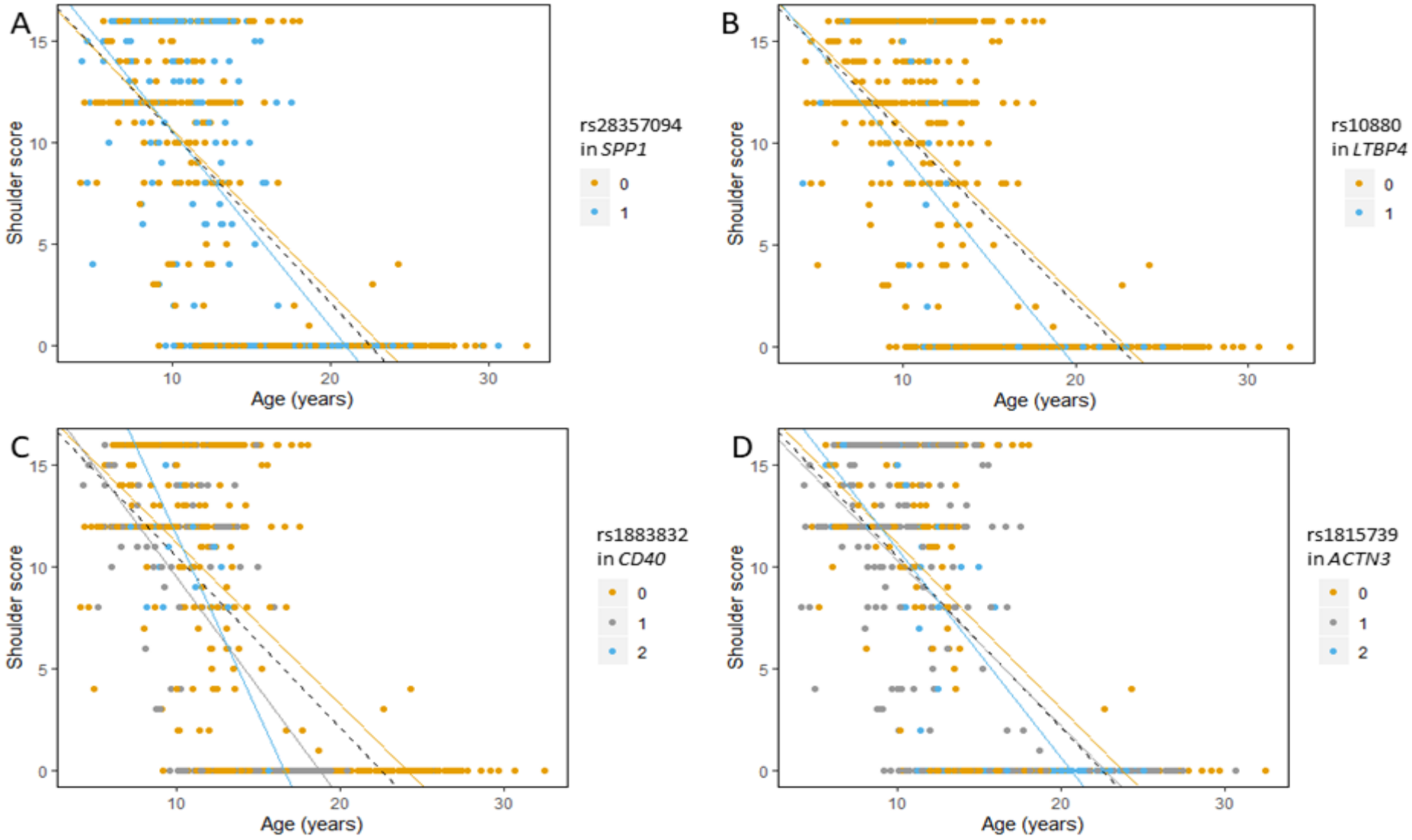


*Figure 18. Scatter plot of Shoulder PUL score by age. Data are grouped by modifiers SNPs genotype; alleles counting is shown based on inheritance model recommended in literature. Regression lines represent the slope of decrease in the linear model. The black regression line describes the whole population.*

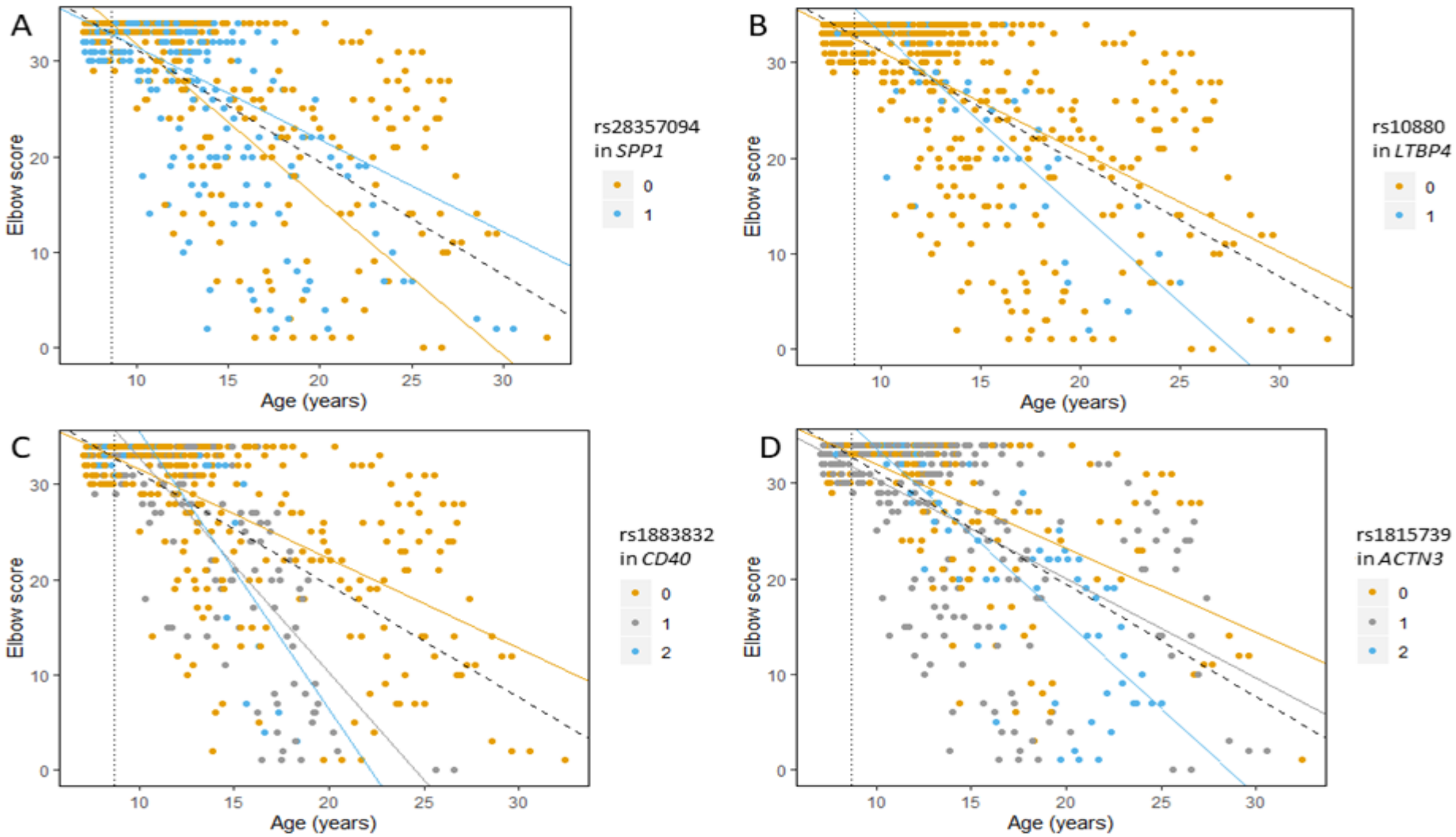


*Figure 19. Scatter plot of Elbow PUL score by age. Data are grouped by modifiers SNPs genotype; alleles counting is shown based on inheritance model recommended in literature. Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model. The black regression line describes the whole population.*

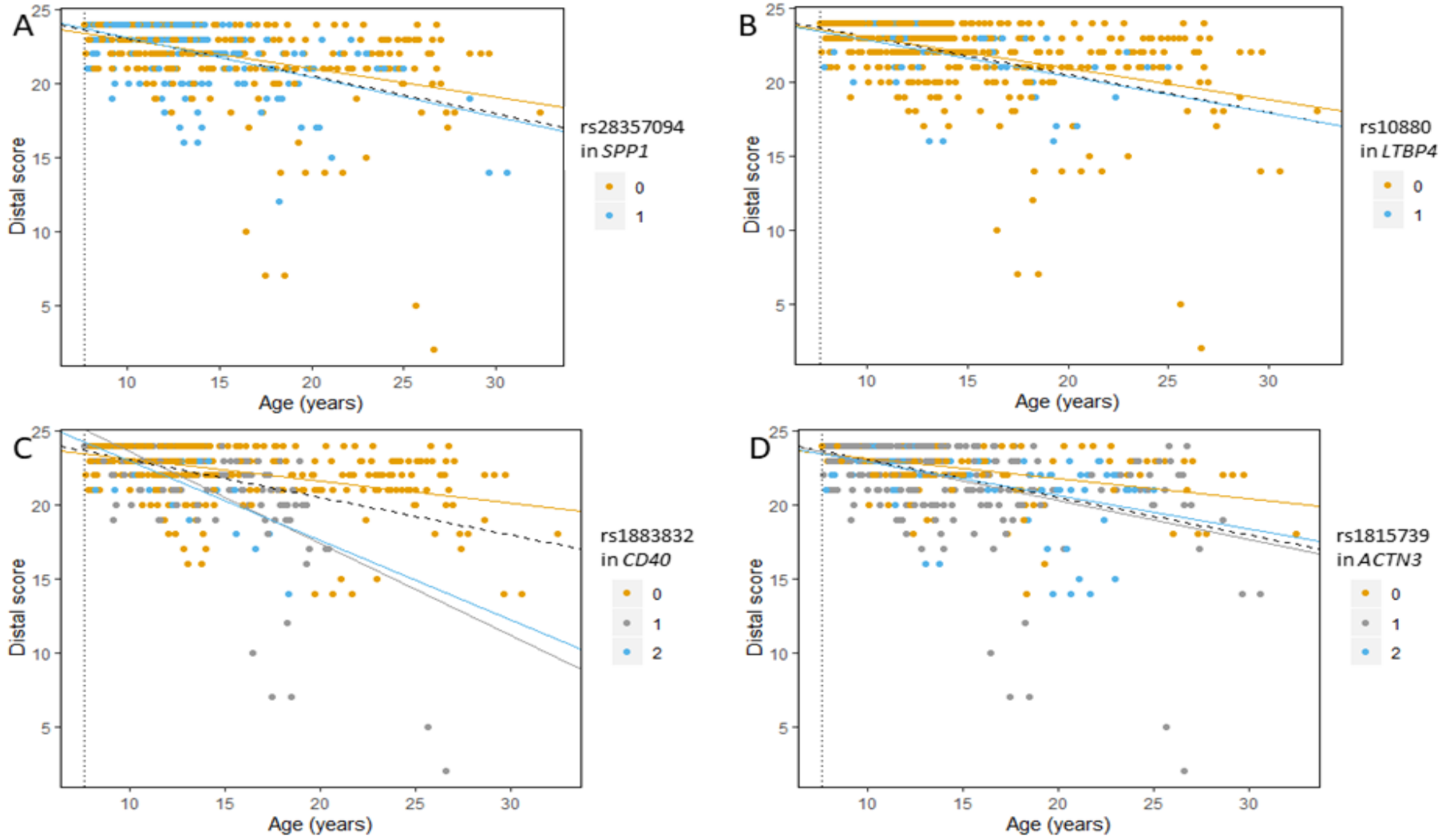


*Figure 20. Scatter plot of Distal PUL score by age. Data are grouped by modifiers SNPs genotype; alleles counting is shown based on inheritance model recommended in literature. Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model. The black regression lines describe the whole population.*

## Discussion

This study confirmed the value of the Performance of Upper Limbs (PUL) scale in the evaluation of DMD patients, both in the ambulatory and non-ambulatory stages of the disease. Nevertheless, we confirmed some test's limitations already described in literature (Pane et al., 2014a). In particular, PUL shows increasing scores in younger patients, and then decreases starting from age of 7. This pattern requires to exclude from the analyses the data not belonging to the linear decrease ranges, especially when applying linear models based on regression, and it is particularly noticeable in Figure 15.

Another drawback is the early decrease of Shoulder score due to proximal weakness that can be found also in ambulatory patients. This leads to large variability in proximal domain scores within the same age class, reducing the statistical power of the Shoulder items (Pane et al., 2014a). This is exemplified in GEE analysis in which GCs treatment effect is not detected, even if its role in DMD pathogenesis is well known (Wong and Christopher, 2002; Angelini et al., 1994; Biggar et al., 2001; Mendell et al., 1989). Furthermore, the power of this analysis was impaired by the fact that there were few untreated patients who retained shoulders function, making the estimate of the linear progression in "natural history" less accurate.

Regarding glucocorticoids treatment, the present study shows that GCs have a protective effect also in non-ambulatory patients, as shown by regression lines in Figure 15 and in (Table 6). Even if this data is strongly significant, we acknowledge a bias due to the retrospective nature of the study. In fact the drug treatment is not randomised and the average age of the untreated population is higher (16.41 ± 6.90) than the treated (12.07 ± 4.68) (Table 3).

With this study we also aimed to describe the natural history of DMD considering different mutations amenable to molecular treatment. A recent study analysed the effects of mutations amenable to skip exon 44, 45, 51 and 53 on loss of ambulation in DMD patients. They found no difference between different skip at 12 months, but after 24 and 36 months it was possible to detect some differences. In particular, patients amenable for exon 53 skip had lower baseline values and more negative changes than the other subgroups, while those amenable to skip exon 44 had better results both at baseline and at follow up, furthermore skip 45 subgroup was associated with a more variable progression (Brogna et al., 2019). Our analysis confirmed these data, but it also detected a protective effect of *DMD* mutations amenable to exon 8 skip, as reported by Wang and colleagues (Wang et al., 2018).

Finally, we analysed the effect of the known modifier SNPs on performance of the upper limbs. Surprisingly we did not find any SNP to have a highly significant effect on PUL score, as only *CD40* showed a nominally significant effect (Table 6). Despite the lack of significance, the effect of the modifiers SNPs follows the expected trends, previously detailed in "Trans" effect – genetic background. For example, it is known from literature that rs1815739 in *ACTN3* is detrimental only

when it is found in heterozygosis (Hogarth et al., 2017), as RR and XX individual have similar performance. Our data showed the same trend as nicely showed in the D panel of Figure 17 to Figure 20.

# Aim 2 - Genetic modifiers of respiratory function in Duchenne muscular dystrophy (Bello et al., 2019. Submitted)

## Introduction

Skeletal muscle weakness and motor disability are the most distinctive clinical features of Duchenne muscular dystrophy; however, respiratory muscle weakness impacts life quality and expectancy even more heavily. Inspiratory/expiratory dysfunction appears around the age of 10, and then progressively deteriorates over the years (LoMauro et al., 2018; McDonald et al., 2018b). Eventually DMD patients develop nocturnal ventilatory insufficiency, requiring non-invasive ventilatory assistance (NIV), and inefficient cough, with reduced airway clearance and increased infection risk. Further disease progression leads to daytime or continuous NIV, sometimes requiring tracheostomy.

As novel DMD treatments - also aiming slowing the progression of respiratory insufficiency, emerge, it is becoming more evident how a quantitative description of its "natural history" is crucial for the design and interpretation of trials. Current standards of care recommend pulmonary function tests (PFTs) yearly in ambulatory patients, and twice yearly in non-ambulatory, as forced vital capacity (FVC) below 1 L or below 50% of the predicted value by age and height (Birnkrant et al., 2018a) indicates a risk of nocturnal hypoventilation.

It has been shown in different DMD cohorts that FVC and other PFT parameters such as peak expiratory flow (PEF) decrease gradually and co-linearly during disease progression, with a tendency to plateau in early and late stages (approximately before the age of 10 or after the age of 20; or above 80% and below 30% of predicted FVC values) (Humbertclaude et al., 2012; LoMauro et al., 2018; McDonald et al., 2018b). As seen for skeletal muscle weakness a striking inter-individual variability is observed in PFT deterioration. In this study we aim to describe the natural history of respiratory insufficiency in DMD, taking in account all the factors that are known to modulate DMD severity (refer to "Phenotype variability" for more details) in a large retrospective cohort followed by the Italian DMD Network. Moreover, as specific *DMD* mutations are now amenable to targeted molecular treatments (Bello and Pegoraro, 2016), such as "exon skipping" antisense oligonucleotides, or small molecules promoting stop codon readthrough, we also described natural history trajectories of these subgroups. Furthermore, as replication of genetic association findings in independent cohorts is crucial (Nelson and Griggs, 2011), we validated findings in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS) cohort.

## Methods

### *Cohorts*

We collected retrospective PFT data at collaborating Italian Centers from January 1990 to May 2018 (henceforth: "Italian cohort"), with the aim a of describing respiratory natural history and GC effects. These have been described elsewhere for CINRG-DNHS cohort (Birnkrant et al., 2018a), which was used here to validate genetic associations.

### *Ethics statement*

All participants or their parents/guardians provided informed consent to study procedures, which were carried out in accordance with the Declaration of Helsinki and approved by Ethics Committees/Institutional Review Boards at participating Institutions.

### *Inclusion criteria*

We selected Italian cohort patients with frameshifting or nonsense *DMD* mutation; absent or <3% dystrophin by immunohistochemistry (IHC, except revertant fibres) or immunoblot; or any *DMD* mutation, plus absent dystrophin as above and/or overt muscle weakness by age 5 years, or loss of independent ambulation by age 13 without GC treatment, or 16 with GCs. CINRG-DNHS criteria were similar (McDonald et al., 2013).

### *PFTs.*

PFTs were performed according to international guidelines (Quanjer et al., 1993), and available measurements of FVC, FEV1 (Forced Expiratory Volume in 1 second), and PEF, expressed as % of predicted, were collected retrospectively. Age and GC treatment status at the time of PFTs, and age at commencement of NIV were collected when available. PFTs in the CINRG-DNHS were performed longitudinally as described (McDonald et al., 2018a).

### *DMD genotype*

Information about pathogenetic *DMD* mutations were collected when available from clinical records or genetic reports. We classified deletions based on amenability to molecular treatments, i.e. skipping of exons 8, 44, 45, 51, and 53 (henceforth: "skip 8", "skip 44", etc.). Nonsense and splice site mutations were also considered as separate groups. Moreover, all mutations were subdivided into "proximal", i.e. situated 5' of intron 44, and therefore not predicted to alter the expression of short dystrophin isoforms (Dp140, Dp116, and Dp71); and "distal", i.e. involving intron 44 and/or regions 3' of it, thus disrupting these isoforms. The same criteria were adopted to classify CINRG-DNHS participants with available DMD mutation data, as described (Bello et al., 2016b).

### *Modifier genotypes*

Patients with available DNA samples were genotyped by TaqMan (Thermo Fisher Scientific) probes at the main known DMD modifier loci: SPP1 rs28357094 (Pegoraro et al., 2011), LTBP4 rs10880 (Flanigan et al., 2013), CD40 rs1883832 (Bello et al., 2016a), and ACTN3 rs1815739 (Hogarth et al., 2017). For tests of genotype/phenotype association, we used the same inheritance models as in published reports. The genotype of modifier SNPs included in the study respect the Hardy-Weinberg equilibrium.

### *Test/validation and "meta-analysis"*

When testing genotype/phenotype associations we used both a test/validation approach, i.e. the same statistical test was performed in the Italian and CINRG-DNHS cohorts; and a meta-analysis approach, i.e. the Italian and CINRG datasets were merged and the same tests were performed. This is justified by the consideration that there is no existing estimate of mutation/SNP effect sizes on the respiratory phenotype, so that formal power calculations are not feasible, leaving the possibility that the test and/or validation cohorts alone may be undersized to identify relevant effects.

### *Statistical analysis*

Quantitative variables were summarized as mean ± standard deviation (SD) and median (range), unless otherwise specified. Intervals of linear decrease of PFT measures were defined on the age axis by piecewise regression, using baseline data (i.e. earliest available value) and choosing a 2-break model for FVC and FEV1, and a 1-break model for PEF after visual inspection of the scatter plot. Generalized Estimating Equations (GEEs) were used to estimate effects of: age; GC treatment (on vs. off at each PFT); DMD mutation (tested separately: each specified mutation group vs. "other" mutations; or "distal" vs. "proximal"); and SNP genotypes (dominant, recessive, or additive as appropriate). GEEs were applied within the "linear" age range defined by piecewise regression, using the same range for test/validation, and recalculating it for meta-analysis. NIV commencement was studied with time-to-event analyses, using age as the time variable. NIV-free participants were censored at last follow-up. Median age at NIV start was estimated with the Kaplan-Meier method. Statistical significance was set at $p < 0.01$ (Bonferroni correction for 5 genetic loci: DMD, SPP1, LTBP4, CD40, ACTN3). No Bonferroni correction was applied to the testing of multiple PFT outcomes as these are strongly intercorrelated and reflect inspiratory/expiratory strength. Statistical analyses were performed using R v.3.5.2.

## Results

### Demographics

For the Italian cohort, we collected data from 1852 PFT evaluations performed by 327 DMD patients, mean age: 11.7±5.3 years. Participants underwent on average 5.7±4.5 evaluations (maximum 19), with intervals of 0.97±0.88 years, with a follow-up time of 4.5±3.9 years (maximum 19.4). At last evaluation, average age was 16.3±6.6 years. Features of the CINRG-DNHS cohort have been described (McDonald et al., 2018b).

### GC treatment

During follow-up, 134 patients (41.0%) were continuously on GC treatment, while 116 (35.5%) were continuously off. Untreated patients were older than treated by approximately 5 years (Table 7). GC treatment in the CINRG-DNHS cohort has been described (McDonald et al., 2018b).

*Table 7. Distribution by glucocorticoid treatment and demographics of treatment subgroups*

| Treatment subgroup | n (%) | Mean age in years ± SD | Median age in years (min - max) |
|---|---|---|---|
| Continuously off GCs | 116 (35.5%) | 14.6±5.9 | 13.7 (3.6 - 44.5) |
| Continuously on GCs | 134 (41.0%) | 9.4±3.5 | 8.6 (4.2 - 24.8) |
| Started GCs during FU | 9 (2.8%) | 6.3±1.4 | 6.3 (3.7 - 8.1) |
| Stopped GCs during FU | 26 (8.0%) | 10.5±3.3 | 9.5 (6.9 - 21.6) |
| Multiple switches | 3 (0.9%) | 12±4.8 | 10.6 (8 - 17.3) |
| Unknown | 39 (11.9%) | 13.0±5.6 | 13.2 (5.5 - 28.0) |
| **Total** | 327 (100%) | 11.7±5.3 | 10.4 (3.6 - 44.5) |

*SD: standard deviation; FU: follow-up; GCS: glucocorticoids; n (%): number of observations.*

### DMD mutations

DMD mutations were defined in 274 (83.8%) patients in the Italian cohort. As expected, single- or multi-exon deletions represented the majority of mutations (70.1%), followed by duplications (11.3%) and nonsense mutations (9.5%). The mutations were “distal” (3’ of intron 44) in 168/274 participants (61.3%), and “proximal” (5’ of intron 44) in 103/274 (37.6%). Three mutations (1.1%) were reported as “nonsense” or “splicing”, but no nucleotide position was available. Distribution by mutations in the CINRG-DNHS was similar, as described previously (Bello et al., 2016b), with 66.9% “distal” and 33.1% “proximal” mutations. The CINRG-DNHS subpopulation with available mutational and PFT data (n=175) is also recapitulated in Table 8.

*Table 8. Distribution by DMD mutation type*

| Mutation group | | Italian cohort n (%) | | CINRG-DNHS cohort n (%) | |
|---|---|---|---|---|---|
| Deletions | skip 8 | 192 (70.1%) | 4 (1.5%) | 138 (78.9%) | 5 (2.9%) |
| | skip 44 | | 16 (5.8%) | | 16 (9.1%) |
| | skip 45 | | 19 (6.9%) | | 22 (12.6%) |
| | skip 51 | | 27 (9.9%) | | 36 (20.6%) |
| | skip 53 | | 25 (9.1%) | | 12 (6.9%) |
| | other | | 101 (36.9%) | | 47 (26.9%) |
| Duplications | | 31 (11.3%) | | 12 (6.9%) | |
| Nonsense mutations | | 26 (9.5%) | | 16 (9.1%) | |
| Small FS mutations | | 13 (4.7%) | | 9 (5.1%) | |
| Splice site mutations | | 10 (3.6%) | | 0 (0.0%) | |
| **Total (molecularly defined)** | | 274 (100%) | | 175 (100%) | |

*"skip 8": deletion amenable to treatment by antisense oligonucleotide promoting the skipping of exon 8; same for other exon numbers. FS: frameshifting.*

## Modifier genotypes

Genotyped SNPs in the Italian cohort showed expected allele frequencies in populations of European ancestry, and Hardy-Weinberg Equilibrium was respected except for a slight violation for ACTN3. Genotype distributions in the CINRG-DNHS cohort have been described (Bello et al., 2015a; Bello et al., 2016a; Hogarth et al., 2017), and did not deviate from HWE Table 9.

*Table 9. Allele frequencies at genotyped modifier loci.*

| Gene and SNP effect | SNP | Italian cohort | | | | | | CINRG-DNHS cohort | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Genotype | | | NA | MAF | HWE deviation | Genotype | | | NA | MAF | HWE deviation |
| | | AA | AB | BB | | | | AA | AB | BB | | | |
| *SPP1* promoter | rs28357094 (T/G) | 115 | 62 | 13 | 137 | 0.23 | n.s. | 186 | 72 | 10 | 9 | 0.17 | n.s. |
| *LTBP4* missense | rs10880 (C/T) | 72 | 85 | 29 | 141 | 0.38 | n.s. | 106 | 129 | 29 | 13 | 0.35 | n.s. |
| *CD40* 5'UTR | rs1883832 (C/T) | 106 | 64 | 8 | 149 | 0.22 | n.s. | 150 | 98 | 19 | 10 | 0.25 | n.s. |
| *ACTN3* nonsense | rs1815739 (C/T) | 39 | 97 | 30 | 161 | 0.47 | p=0.03 | 74 | 114 | 70 | 19 | 0.49 | n.s. |

*AA: major allele homozygote. AB: heterozygote. BB: minor allele homozygote. NA: undetermined genotype (DNA unavailable or insufficient). MAF: minor allele frequency.*

## Ranges of linear decrease.

Using piecewise regression, we estimated in the Italian cohort that expiratory volumes decreased linearly from the age of 8.6 (FVC) or 8.5 (FEV1), to the age of 22.7 years; while PEF started decreasing linearly since the earliest PFTs, until the age of 27.1 (Figure 21 - Figure 23). In the "meta-analysis"

cohort the boundaries of linear decrease were similar: 8.7 to 22.6 years for FVC, 8.8 to 19.0 for FEV1, 0 to 25.4 for PEF (Table 10).

### Effects of age and GCs

In the Italian cohort, the GEE model estimated the rate of yearly decline (± standard error) of FVC as -4.2±0.2% of predicted, while the decline of FEV1 was -5.0±0.3%, and that of PEF -2.9±0.3%. GC treatment was associated to increased FVC (4.6±2.1%), FEV1 (15.5±2.0%), and PEF (14.2±1.9). As visualized by the regression lines in Figure 21, Figure 22 and Figure 23, GC treatment was associated with higher PFT measures since young ages, but with similar declining slopes over the years. Decline rates of FVC and FEV1 in the CINRG-DNHS were similar (within 0.6%), while the decline of PEF appeared somewhat faster (-3.8±0.2%) (Table 10).

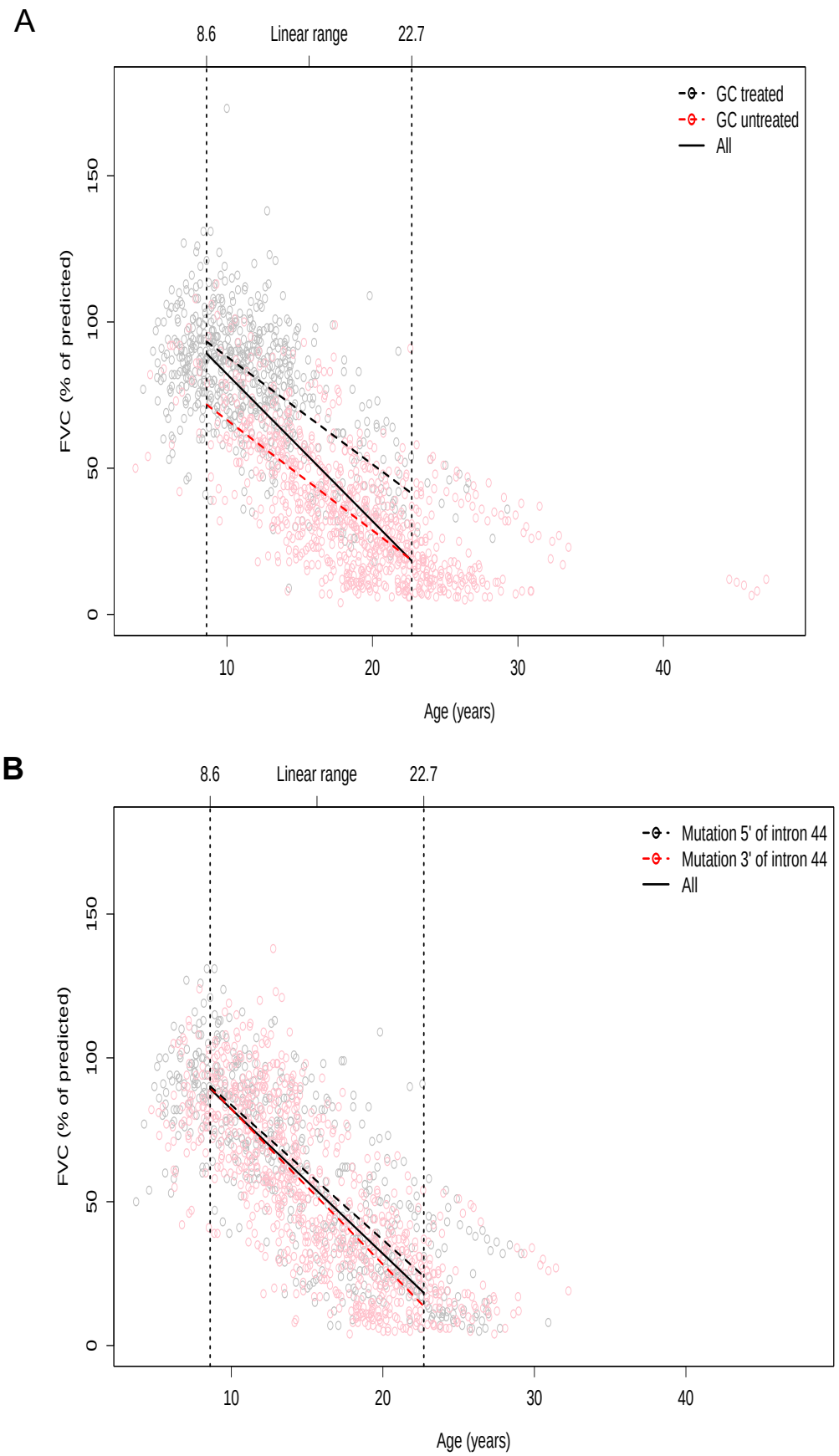


*Figure 21. Scatter plots of FVC by age in the Italian cohort, grouped by GC treatment at the time of spirometry (A) and by DMD mutation type ("proximal" or "distal") (B). Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model.*

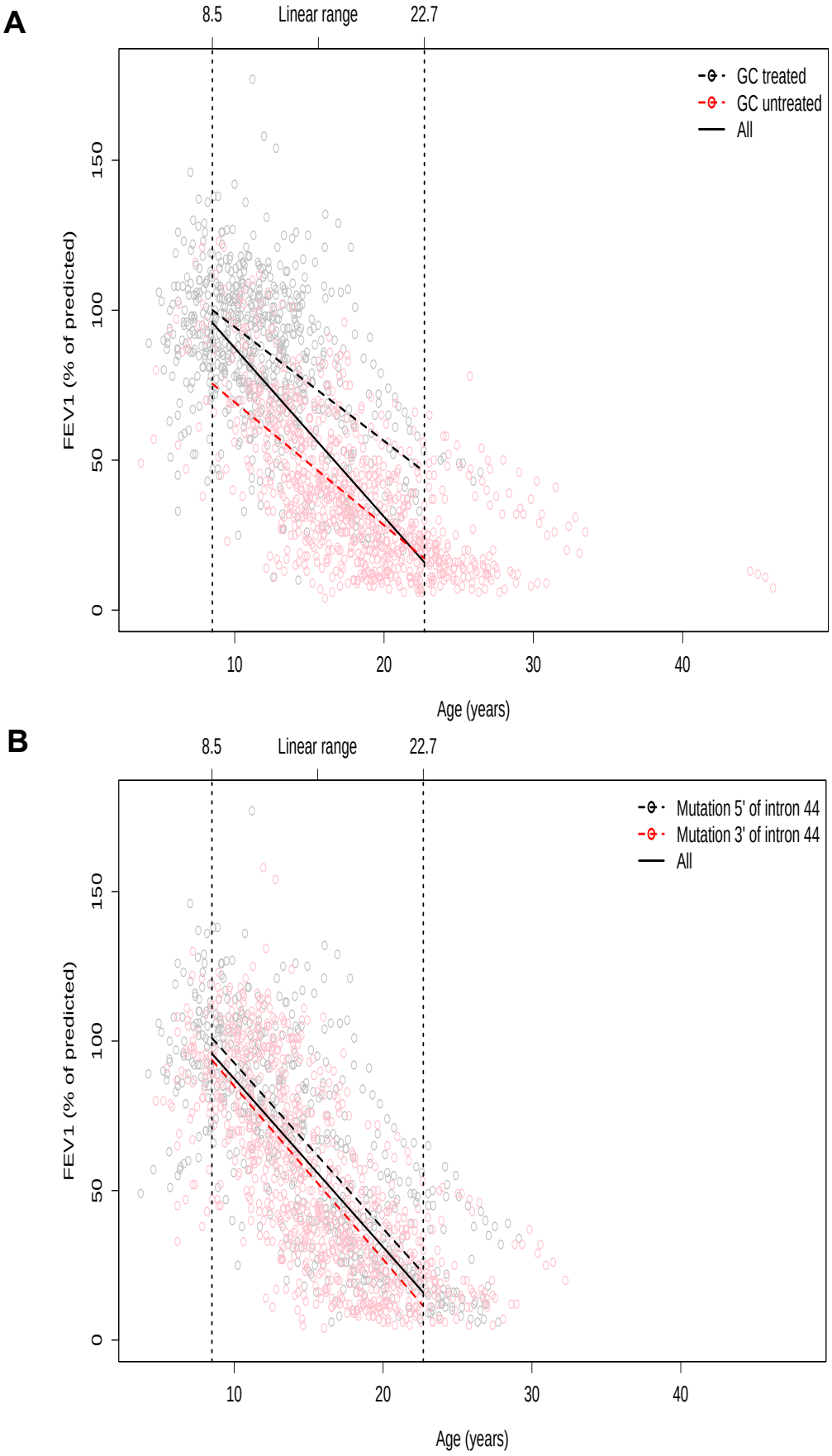


*Figure 22. Scatter plots of FEV1 by age in the Italian cohort, grouped by GC treatment at the time of spirometry (A) and by DMD mutation type ("proximal" or "distal" (B). Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model.*

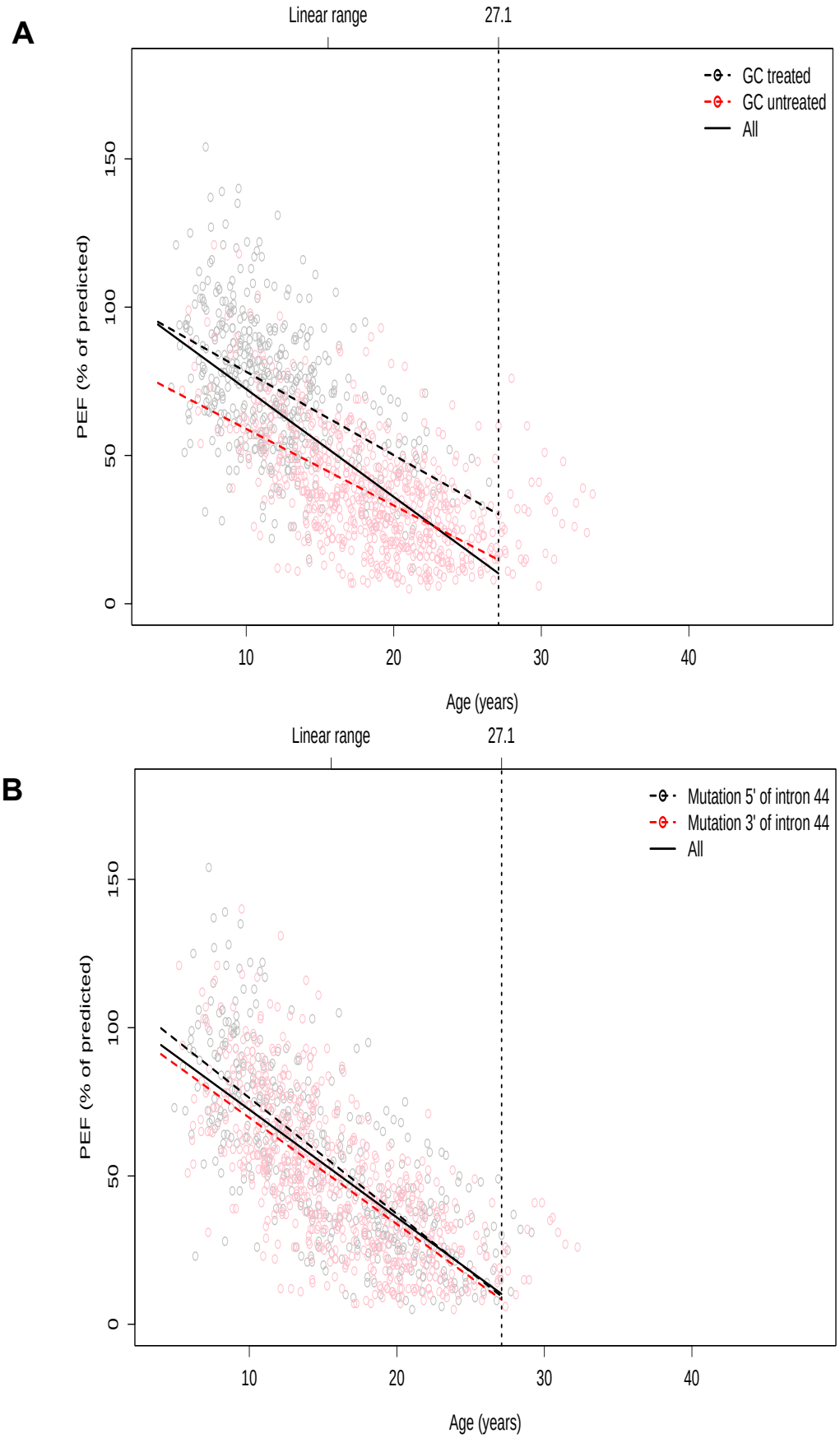


*Figure 23. Scatter plots of PEF by age in the Italian cohort, grouped by GC treatment at the time of spirometry (A) and by DMD mutation type ("proximal" or "distal") (B). Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model.*

## DMD mutation effects

In the Italian cohort, “distal” mutations downstream of exon 44 were significantly associated with lower FVC (-6.1±2.3%), and with lower FEV1 (-6.3±2.5%) and PEF (5.8±2.3%), with significance bordering the Bonferroni-corrected threshold. Validation in the CINRG cohort showed similar estimates with nominal significance for FVC and FEV1. With the meta-analysis approach, the negative effect of distal mutations showed p-values of 0.001 (FVC), 0.0094 (FEV1), and 0.013 (PEF). FVC was negatively correlated with “skip 51” and “skip 53” mutations, the latter with large effect sizes (approximately -10%) in all cohorts. “Skip 44” mutations showed nominally significant increases of FVC with the meta-analysis approach (+7.1±3.3%, p-value = 0.016). “Skip 8” mutations were associated with dramatic increases of PEF (+20.0±4.5%) and nominally significant increases of FVC (+13.8±8.3%) and FEV1 (+15.3±7.9%) (Table 10). Interestingly, splice site mutations were associated with higher expiratory volumes in the Italian cohort (nominally significant; no such mutations were reported in the CINRG-DNHS). FVC and PEF trajectories associated to different *DMD* and SNP genotypes (merged meta-analysis cohort) are shown in Figure 24 and Figure 25.

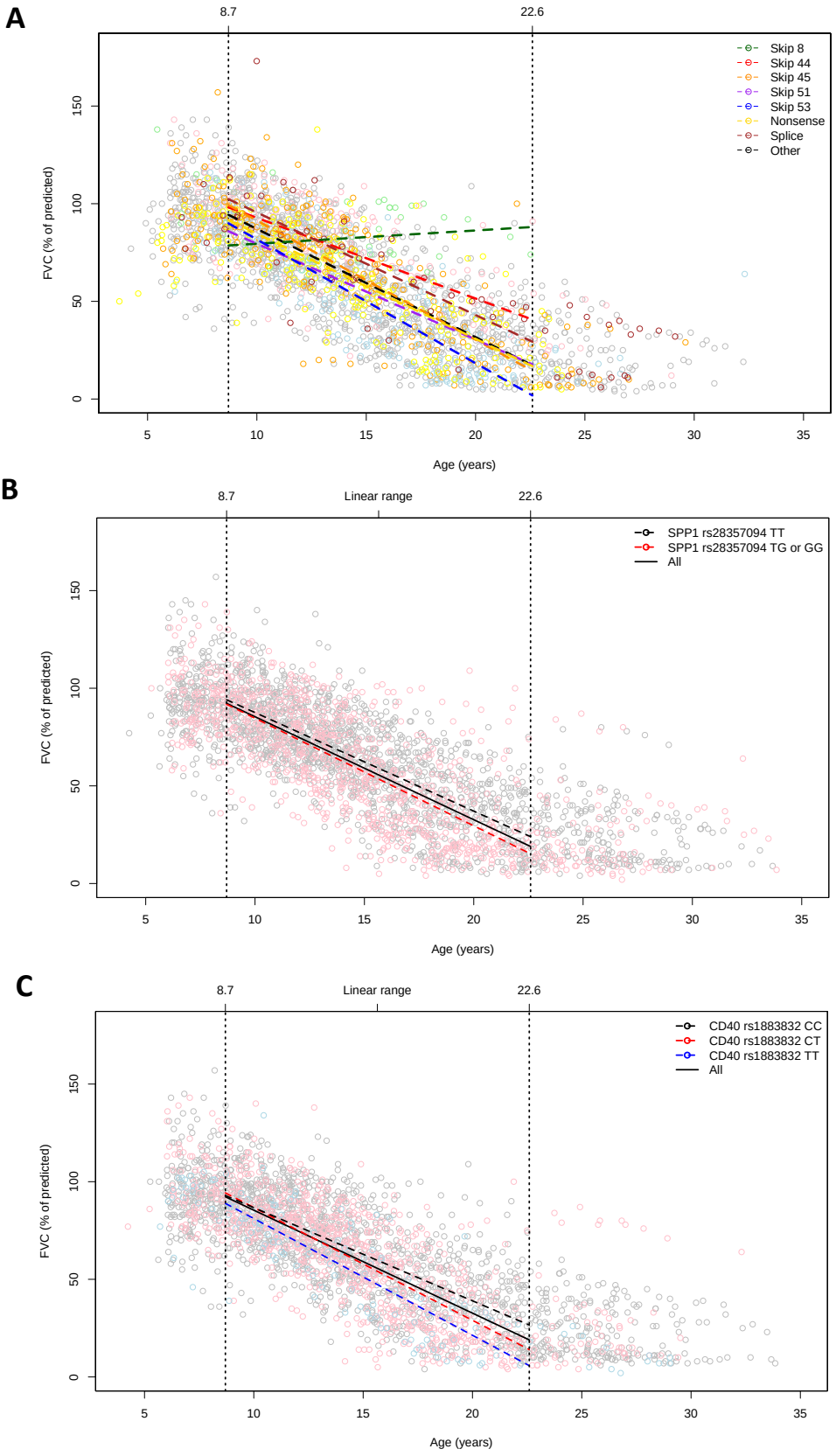


*Figure 24. Scatter plots of FVC by age in the merged Italian and CINRG-DNHS cohorts, grouped by DMD mutation type (groups defined in Methods) (A) and by modifier SNP genotypes (SPP1 rs28357094, panels C; CD40 rs1883832, panels C). Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model.*

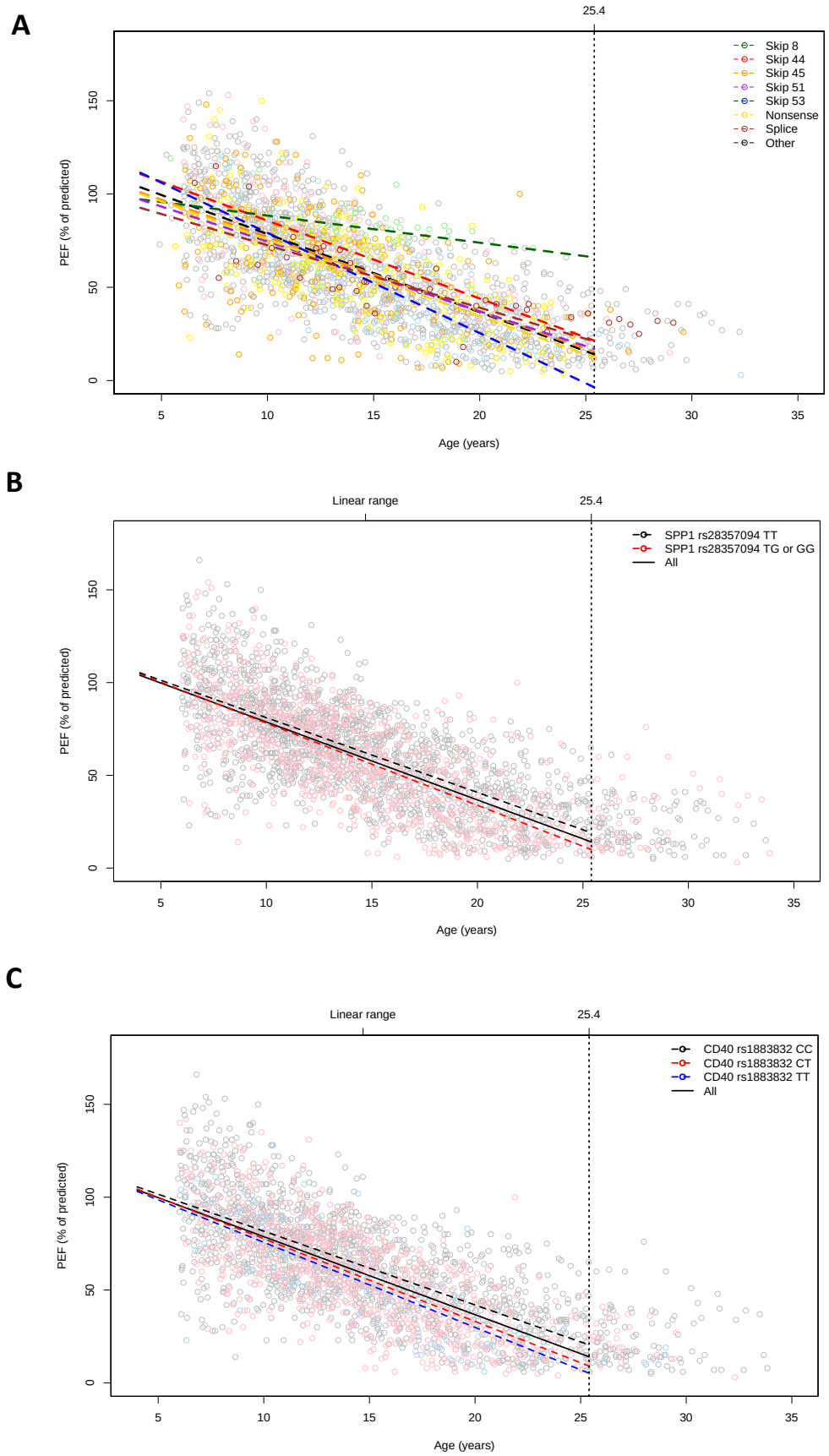


*Figure 25. Scatter plots of PEF by age in the merged Italian and CINRG-DNHS cohorts, grouped by DMD mutation type (groups defined in Methods) (A) and by modifier SNP genotypes (SPP1 rs28357094, panels C; CD40 rs1883832, panels C). Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model.*

## SNP effects

The dominant G genotype at rs28357094 in the SPP1 promoter, associated with earlier LoA in DMD (Bello et al., 2012; Bello et al., 2015a; Pegoraro et al., 2011), showed a nominally significant negative effect on FVC (-4.5±2.5%) in the meta-analysis. A significant effect on PEF was observed in the CINRG (-8.7±3.1%, p-value = 0.005) and meta-analysis (-6.3±2.4%, p-value = 0.0088) cohorts. The additive T genotype at rs1883832 in the CD40 5' untranslated region (UTR), also causing earlier LoA (Flanigan et al., 2013), showed a significant negative effect on FVC in the CINRG (-6.1±2.2%, p-value = 0.005) and meta-analysis (-4.8±1.7%, p-value = 0.006) cohorts; and nominally significant effects on FEV1 in the CINRG-DNHS, and on PEF in the meta-analysis cohort. LTBP4 and ACTN3 SNPs showed no relevant associations with PFTs (Table 10).

*Table 10. Coefficients of Generalized Estimating Equation (GEE) analyses.*

| **FVC (% predicted)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Coefficient** | | **Italian cohort** | | **CINRG-DNHS cohort** | | **Meta-analysis** | |
| | | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** |
| **Intercept** | | 111.8 ± 3.9 | **< 0.0001** | 125.0 ± 4.7 | **< 0.0001** | 119.4 ± 3.0 | **< 0.0001** |
| **Age (per-year decrease)** | | -4.2 ± 0.2 ↓ | **< 0.0001** | -4.8 ± 0.3 ↓ | **< 0.0001** | -4.6 ± 0.2 ↓ | **< 0.0001** |
| **GC treatment** | | 14.5 ± 2.1 ↑↑ | **< 0.0001** | 9.4 ± 2.3 ↑ | **< 0.0001** | 11.7 ± 1.6 ↑↑ | **< 0.0001** |
| **Mutation 3' of exon 44** | | -6.1 ± 2.3 ↓ | **0.008** | -5.9 ± 2.7 ↓ | 0.029 | -5.8 ± 1.8 ↓ | **0.001** |
| **Mutation type** | **Skip 8** | 12.5 ± 8.9 | n.s. | 13.2 ± 12.0 | n.s. | 13.8 ± 8.3 ↑↑ | 0.049 |
| | **Skip 44** | 6.5 ± 5.0 | n.s. | 7.8 ± 4.5 | 0.04 | 7.1 ± 3.3 ↑ | 0.016 |
| | **Skip 45** | -3.4 ± 5.2 | n.s. | 0.1 ± 3.3 | n.s. | -0.8 ± 2.8 | n.s. |
| | **Skip 51** | -6.9 ± 3.8 ↓ | 0.035 | -5.7 ± 3.1 ↓ | 0.031 | -5.7 ± 2.3 ↓ | **0.007** |
| | **Skip 53** | -9.2 ± 3.3 ↓ | **0.003** | -11.6 ± 4.7 ↓↓ | **0.007** | -10.3 ± 2.7 ↓↓ | **< 0.0001** |
| | **Nonsense** | 2.3 ± 3.2 | n.s. | -6.3 ± 4.0 | 0.059 | -1.1 ± 2.6 | n.s. |
| | **Splice site** | 13.9 ± 7.0 ↑ | 0.023 | NA | NA | 13.6 ± 7.0 ↑ | 0.069 |
| **SNP modifiers** | **rs28357094 dom** | -5.3 ± 3.8 | n.s. | -4.4 ± 3.2 | n.s. | -4.5 ± 2.5 ↓ | 0.020 |
| | **rs10880 rec** | -6.8 ± 4.2 | n.s. | 3.4 ± 3.6 | n.s. | -1.4 ± 2.8 | n.s. |
| | **rs1883832 add** | -0.1 ± 2.9 | n.s. | -6.1 ± 2.2 ↓ | **0.005** | -4.8 ± 1.7 ↓ | **0.006** |
| | **rs1815739 add** | 4.2 ± 2.4 | n.s. | 0.3 ± 1.7 | n.s. | 0.8 ± 145 | n.s. |
| **FEV1 (% predicted)** | | | | | | | |
| **Coefficient** | | **Italian cohort** | | **CINRG-DNHS cohort** | | **Meta-analysis** | |
| | | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** |
| **Intercept** | | 125.8 ± 4.3 | **< 0.0001** | 122.6 ± 4.6 | **< 0.0001** | 127.0 ± 3.9 | **< 0.0001** |
| **Age (per-year decrease)** | | -5.0 ± 0.3 ↓ | **< 0.0001** | -4.6 ± 0.3 ↓ | **< 0.0001** | -5.1 ± 0.2 ↓ | **< 0.0001** |
| **GC treatment** | | 15.1 ± 2.0 ↑↑ | **< 0.0001** | 8.5 ± 2.4 ↑ | **0.0003** | 13.4 ± 1.7 ↑↑ | **< 0.0001** |
| **Mutation 3' of exon 44** | | -6.3 ± 2.5 ↓ | 0.011 | -6.6 ± 2.9 ↓ | 0.025 | -5.3 ± 2.1 ↓ | **0.0094** |
| **Mutation type** | **Skip 8** | 9.7 ± 7.5 | n.s. | 17.9 ± 11.4 | 0.058 | 15.3 ± 7.9 ↑↑ | 0.027 |
| | **Skip 44** | 0.9 ± 6.0 | n.s. | 7.0 ± 4.5 | 0.059 | 3.2 ± 4.0 | n.s. |
| | **Skip 45** | -0.7 ± 5 | n.s. | -2.7 ± 4.4 | n.s. | -1.9 ± 3.3 | n.s. |
| | **Skip 51** | -7.5 ± 4.3 ↓ | 0.042 | -4.8 ± 3.4 | n.s. | -5.3 ± 2.9 ↓ | 0.032 |
| | **Skip 53** | -6.1 ± 3.8 | 0.053 | -10.9 ± 4.7 ↓↓ | 0.010 | -6.6 ± 3.2 ↓ | 0.021 |
| | **Nonsense** | 2.7 ± 4.5 | n.s. | -7.1 ± 4.3 ↓ | 0.048 | -1.5 ± 3.6 | n.s. |
| | **Splice site** | 5.8 ± 4.3 | n.s. | NA | NA | 11.6 ± 5.1 ↑↑ | 0.011 |
| **SNP modifiers** | **rs28357094 dom** | -3.3 ± 4.2 | n.s. | -5.8 ± 3.6 | 0.055 | -2.9 ± 2.9 | n.s. |
| | **rs10880 rec** | -4.3 ± 5.2 | n.s. | 5.4 ± 3.8 | n.s. | -2.6 ± 1.9 | n.s. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **rs1883832 add** | 2.0 ± 3.1 | n.s. | -4.8 ± 2.2 ↓ | 0.030 | -2.1 ± 3.2 | n.s. |
| | **rs1815739 add** | 1.3 ± 3.2 | n.s. | 1.2 ± 1.8 | n.s. | 0.6 ± 1.7 | n.s. |
| **PEF (% predicted)** | | | | | | | |
| **Coefficient** | | **Italian cohort** | | **CINRG-DNHS cohort** | | **Meta-analysis** | |
| | | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** |
| **Intercept** | | 89.3 ± 3.6 | **< 0.0001** | 109.4 ± 4.6 | **< 0.0001** | 90.7 ± 3.7 | **< 0.0001** |
| **Age (per-year decrease)** | | -2.9 ± 0.2 ↓ | **< 0.0001** | -3.8 ± 0.2 ↓ | **< 0.0001** | -3.0 ± 0.2 ↓ | **< 0.0001** |
| **GC treatment** | | 14.2 ± 1.9 ↑↑ | **< 0.0001** | 8.0 ± 1.9 ↑ | **< 0.0001** | 14.1 ± 1.9 ↑↑ | **< 0.0001** |
| **Mutation 3' of exon 44** | | -5.8 ± 2.3 ↓ | 0.010 | -4.7 ± 2.8 | n.s. | -5.6 ± 2.3 ↓ | 0.013 |
| **Mutation type** | **Skip 8** | 23.0 ± 4.2 ↑↑ | **< 0.0001** | 16.1 ± 6.4 ↑↑ | **0.006** | 20.0 ± 4.5 ↑↑ | **< 0.0001** |
| | **Skip 44** | -0.6 ± 5.4 | n.s. | 5.9 ± 4.2 | n.s. | 3.9 ± 3.5 | n.s. |
| | **Skip 45** | -7.8 ± 5.2 | n.s. | -0.7 ± 4.4 | n.s. | -2.9 ± 3.4 | n.s. |
| | **Skip 51** | -5.7 ± 3.4 ↓ | 0.048 | -2.2 ± 3.8 | n.s. | -2.9 ± 2.6 | n.s. |
| | **Skip 53** | -5.5 ± 2.9 ↓ | 0.027 | -5.3 ± 5.2 | n.s. | -5.9 ± 2.7 ↓ | 0.014 |
| | **Nonsense** | 0.5 ± 2.8 | n.s. | -6.0 ± 5.1 | n.s. | -2.3 ± 3.0 | n.s. |
| | **Splice site** | -0.9 ± 5.1 | n.s. | NA | NA | -3.0 ± 5.1 | n.s. |
| **SNP modifiers** | **rs28357094 dom** | -2.3 ± 3.5 | n.s. | -8.7 ± 3.1 ↓ | **0.005** | -6.3 ± 2.4 ↓ | **0.0088** |
| | **rs10880 rec** | -6.2 ± 4.0 | n.s. | 4.9 ± 4.1 | n.s. | 1.3 ± 3.1 | n.s. |
| | **rs1883832 add** | -3.7 ± 3.0 | n.s. | -3.0 ± 2.2 | n.s. | -4.1 ± 1.8 ↓ | 0.024 |
| | **rs1815739 add** | 4.0 ± 2.5 | n.s. | 0.1 ± 1.8 | n.s. | 1.2 ± 1.5 | n.s. |

*SE: Standard Error. GC: glucocorticoid corticosteroids. Skip 8: mutations amenable to treatment with skipping of exon 8 (same for other exon numbers). NA: not available (no participants included in the corresponding category). n.s.: not significant. Nominally significant effects have been marked with arrows, upward for positive effects and downward for negative. Double arrows indicate "strong" effects (arbitrarily: above 10 % of the corresponding measure). p-values < 0.06 (nominally significant or close) are indicated in numbers; p-values < 0.01 are highlighted in **bold**.*

## NIV

NIV was initiated in 87/318 participants with available data in the Italian cohort, and 31/276 in the CINRG-DNHS, at a median age of 23.2 and 22.2 years respectively. The effect of GC treatment in delaying NIV was estimated around 2 years in the Italian cohort (p-value = n.s.), not validated in the CINRG-DNHS. There was no clear effect of DMD mutation type on age at NIV. A nominally significant effect was observed for SPP1 rs28357094 with the meta-analysis approach, with an HR of 1.75 (detrimental). For CD40 rs1883832, there were nominally significant effects in the CINRG-DNHS and meta-analysis cohorts (HR 1.71 and 1.50, p-values = 0.044 and 0.0498 respectively) (Table 11). Cumulative incidence plots for suggestive associations are illustrated in Figure 26.

*Table 11. Coefficients of time-to-event analyses of age at commencement of NIV.*

| | | Italian cohort | | | | CINRG-DNHS cohort | | | | Meta-analysis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **n (events)** | **Median age at NIV (95% CI)** | **HR (95% CI)** | **p-value** | **n (events)** | **Median age at NIV (95% CI)** | **HR (95% CI)** | **p-value** | **n (events)** | **Median age at NIV (95% CI)** | **HR (95% CI)** | **p-value** |
| **All participants** | | 318 (87) | 23.2 (22.0-24.0) | NA | NA | 276 (31) | 22.2 (21.8-NA) | NA | NA | 594 (118) | 23.0 (22.0-24.0) | NA | NA |
| **GC treatment** | **Treated** | 166 (15) | 24.0 (23.0-NA) | 0.67 (0.37-1.18) | n.s. | 211 (23) | 22.2 (22.1-NA) | 0.91 (0.41-2.06) | n.s. | 377 (38) | 24.0 (22.2-NA) | 0.79 (0.53-1.18) | n.s. |
| | **Untreated** | 113 (62) | 22.0 (20.0-24.0) | | | 65 (8) | 22.0 (21.8-NA) | | | 178 (70) | 22.4 (21.0-24.0) | | |
| ***DMD* mutation** | **5' exon 44** | 102 (24) | 23.0 (21.0-NA) | 0.98 (0.59-1.60) | n.s. | 58 (3) | NA (17.2-NA) | 0.76 (0.21-2.71) | n.s. | 160 (27) | 23.2 (21.0-NA) | 0.92 (0.59-1.46) | n.s. |
| | **3' exon 44** | 168 (47) | 22.9 (20.0-24.0) | | | 117 (13) | 21.8 (20.0-NA) | | | 285 (60) | 22.9 (20.0-24.0) | | |
| ***SPP1* rs28357094 (dominant)** | **TT** | 110 (20) | 28.0 (25.0-NA) | 1.72 (0.91-3.26) | 0.094 | 185 (15) | 25.0 (22.2-NA) | 1.71 (0.80-3.66) | n.s. | 295 (35) | 27.0 (25.0-NA) | 1.75 (1.08-2.84) | 0.023 |
| | **TG or GG** | 72 (19) | 22.9 (22.0-NA) | | | 82 (13) | 20.0 (17.4-NA) | | | 154 (32) | 22.0 (20.0-24.0) | | |
| ***LTBP4* rs10880 (recessive)** | **CC or CT** | 149 (31) | 24.0 (23.0-30.0) | 1.27 (0.58-2.79) | n.s. | 234 (29) | 22.2 (21.8 -NA) | 0.35 (0.05-2.59) | n.s. | 383 (60) | 24.0 (22.2-28.0) | 0.91 (0.45-1.85) | n.s. |
| | **TT** | 29 (8) | 23.0 19.2-NA) | | | 29 (1) | NA (NA-NA) | | | 58 (9) | 30.9 (23.0-NA) | | |
| ***CD40* rs1883832 (additive)** | **CC** | 99 (23) | 24.0 (22.0-NA) | 0.97 (0.47-1.99) | n.s. | 150 (11) | 25.1 (22.0-NA) | 1.71 (1.01-2.88) | 0.044 | 249 (34) | 24.0 (22.9-NA) | 1.50 (1.00-2.26) | 0.0498 |
| | **CT** | 63 (10) | 27.0 (22.0-NA) | | | 98 (16) | 20.0 (17.0-NA) | | | 161 (26) | 23.0 (20.0-NA) | | |
| | **TT** | 8 (0) | NA (NA-NA) | | | 18 (3) | 21.4 (16.4-NA) | | | 26 (3) | 26.0 (16.8-NA) | | |
| ***ACTN3* rs1815739 (additive)** | **CC** | 37 (7) | 29.0 (19.0-NA) | 1.10 (0.58-2.10) | n.s. | 74 (5) | NA (20.0-NA) | 0.26 (0.69-1.92) | n.s. | 111 (12) | 29.0 (20-NA) | 1.18 (0.79-1.78) | n.s. |
| | **CT** | 92 (12) | 27.0 (23.2-NA) | | | 114 (15) | 22.2 (21.8-NA) | | | 206 (27) | 27.0 (23-NA) | | |
| | **TT** | 29 (6) | 22.9 (20.0-NA) | | | 69 (7) | 25.0 (22.1-NA) | | | 98 (13) | 22.9 (20.0-NA) | | |

*n (events): number of participants included in the analysis and (in brackets) number of observed events, i.e. commencement of NIV. CI: Confidence Interval. Age is indicated in years. HRs above 1 indicate detrimental effects (i.e. earlier NIV), while HRs below 1 correspond to later NIV. The direction of HRs is calculated for GC treated vs. untreated; "distal" vs. "proximal" DMD mutation; and minor alleles for modifier SNPs (with the indicated inheritance models).*

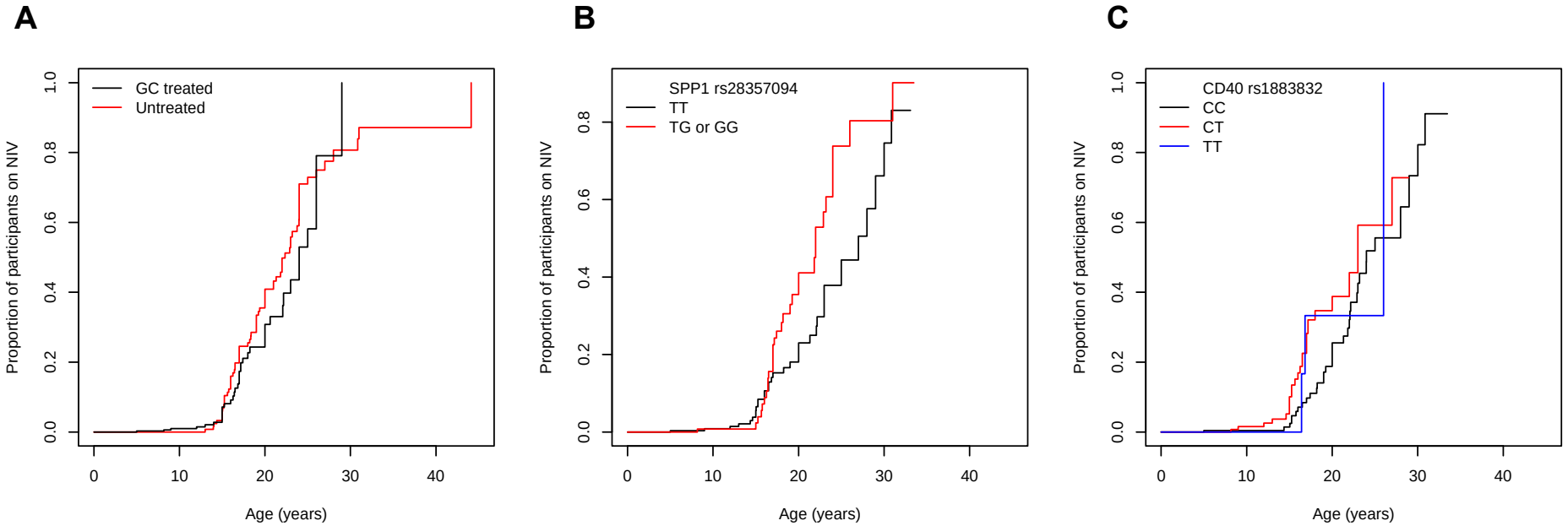


*Figure 26. Cumulative incidence plots of NIV use by age in the merged Italian and CINRG-DNHS cohorts, grouped by (A) GC treatment (on vs. off during follow-up), (B) SPP1 rs28357094 genotype, and (C) CD40 rs1883832 genotype*

## Discussion

In this study we quantitatively described the progressive reduction of respiratory function measurements in a large cohort of Italian DMD patient. The estimation of yearly rates of decline for these measurements have a great relevance to both the clinical management of patients, as they allow to gauge the severity of individual trajectories of respiratory decline compared to the general DMD population, and for the design and interpretation of clinical trials based on PFT endpoints.

The FVC decline slope identified in the Italian cohort (-4.2%/year) is in line with several previous reports (Mayer et al., 2015; McDonald et al., 2018b; Ricotti et al., 2019) which estimated values of decline around 5%/year for both FVC and PEF. The decline of PEF, on the other hand, appeared somewhat slower in the Italian cohort (-2.9%/year). This may be partly due to methodological aspects of our study, in which we applied linear models only within the linear age range of decline estimated by piecewise regression. PEF appeared to decline linearly over a considerably longer time than FVC and FEV1 in the Italian cohort, and the yearly decline rate appeared subsequently smaller. In fact, the application of piecewise regression algorithms to our dataset provide useful information for the design of incusion/exclusion criteria for future trials focusing on respiratory endpoints in DMD, which ideally aim at recruiting participants in a linear decline phase. For instance, we suggest that PEF may be a more sensitive primary endpoint in studies involving younger boys or older adults (approximately < 10 or > 20 years of age), while FVC remains the most sensitive outcome in the intermediate age range.

We confirm a large effect of GCs in improving respiratory function in DMD. We recognize potential sources of bias which might lead to a partial over-estimation of the GC effect: first, the treated population was younger, and might have benefited from an improvement of overall standards of care. Second, percent-predicted PFT values are influenced by height (denominator in the formula), and patients treated chronically with GC display stunted growth (Matthews et al., 2016). However, the beneficial effect of GCs appears uncontroversial. Interestingly, treated and untreated participants show similar decline slopes (Figure 21 A, Figure 22 A, Figure 23 A), but starting from a higher plateau in treated. This advantage is maintained over time, delaying the age at which functional thresholds related to nocturnal and diurnal respiratory insufficiency are reached. Overall, our findings support continuation of GC treatment throughout the non-ambulatory phases of DMD, at doses progressively tailored to individual tolerability profiles (usually lower than those recommended at start of treatment) (Birnkrant et al., 2018b; Gloss et al., 2016).

An unexpected finding was the detrimental effect on PFTs of “distal” DMD mutations, potentially affecting the expression of short dystrophin isoforms. The association in the Italian cohort was consistently validated in the CINRG-DNHS, with an effect size of around 6% for FVC and PEF. The restrictive ventilatory defect in DMD is caused by the absence of full-length dystrophin in the

diaphragm and other respiratory muscles. While defects of short dystrophin isoforms have been associated with increased risk of mental retardation and other central nervous system (CNS) manifestations of DMD (Doorenweerd et al., 2017; Felisari et al., 2000; Magri et al., 2011), they have not been clearly linked to more severe muscle weakness. A possible explanation is that although patients with very severe cognitive deficiencies are excluded from PFTs, because of insufficient collaboration, patients with subtler CNS issues due to Dp140/Dp71 defects still perform worse in PFTs, which are largely influenced by volition and effort. However, an influence of short dystrophin isoforms on other physiological variables, such as chest wall compliance or skeletal deformities, may not be ruled out entirely, and warrants further studies.

When looking into mutations amenable to different molecular treatments (Bello and Pegoraro, 2016), the main anticipated finding of a milder phenotype in participants with “skip 44” deletions (Bello et al., 2016b; Pane et al., 2014b; van den Bergen et al., 2014; Wang et al., 2018) appeared only marginal, and only nominally significant for FVC in the meta-analysis. Possibly because of the relatively small size of these subgroups (n=16 in the Italian and 16 in the CINRG cohort). Even with regards to ambulatory function, it should be noted, the modifier effect of “skip 44” mutations was in fact variable (Bello et al., 2016b; Pane et al., 2014b; van den Bergen et al., 2014; Wang et al., 2018). Proposed underlying mechanisms involve alternative splicing of exon 44 by activation of cryptic splice sites at the deletion breakpoint (Dwianingsih et al., 2014), which may vary between individuals.

Conversely, the “skip 51” and especially “skip 53” subgroups surprisingly showed worse PFT outcomes (especially PEF) across the Italian and CINRG cohorts. This finding is relevant to ongoing clinical trials of exon skipping drugs such as eteplirsen, which was recently approved in the USA (Aartsma-Rus and Krieg, 2017) and shown to modify slopes of FVC change compared to the genotyped CINRG cohort (Khan et al., 2019). “Skip 51” and “skip 53” mutations are completely included in the “distal” group, and predicted to disrupt Dp140 expression. Therefore, these participants may fare worse in PFTs because of the reasons proposed above.

The four modifier SNPs tested here have been described because of their effect on muscle strength, and mainly ambulatory function (Bello et al., 2012; Bello et al., 2015a; Bello et al., 2016a; Flanigan et al., 2013; Hogarth et al., 2017; Pegoraro et al., 2011; van den Bergen et al., 2015). Respiratory involvement is more severe in DMD patients with more severe skeletal muscle impairment (Humbertclaude et al., 2012); thus, modifiers of skeletal muscle strength may be considered candidate modifiers of respiratory insufficiency. In fact, similar inflammatory, regenerative, and fibrotic events occur in the diaphragm as in skeletal muscle, although with potential differences (Rouger et al., 2002), and may be modulated by modifier genes. In this study, no loci validated independently across the two cohorts with Bonferroni-corrected significance. Meta-analysis identified suggestive effects of

SPP1 and CD40 on FVC and PEF, in the expected direction (minor alleles being detrimental). Both the SPP1-encoded cytokine osteopontin (Pagel et al., 2014) and T-cell activation mediated by CD40 (Rosenberg et al., 2015) preferentially influence the earlier inflammatory phase of the dystrophic process, rather than the late, end-stage fibrotic phase (Chen et al., 2005b) The latter is more affected by latent transforming growth factor β-binding protein LTBP4 (Quattrocelli et al., 2017), whose disease-modifying polymorphisms did not show significant effects on PFTs. For LTBP4, this result did not change when taking into consideration the full VTTT/IAAM haplotype (Flanigan et al., 2013). These findings suggest that active dystrophic pathology with necrosis and inflammation of respiratory may be more relevant to PFT measures than the degree of chronic fibrosis. The clinical meaningfulness of SPP1 and CD40 effects was strengthened by findings of earlier NIV associated with risk variants, although only nominally significant.

We acknowledge several limitations to this study: the retrospective nature of Italian cohort data, compensated for by validation in the longitudinal CINRG-DNHS (McDonald et al., 2018b); the unavailability of maximal inspiratory and expiratory pressure (MIP and MEP) data, which we plan to collect in future studies; the concern that some missing data regarding NIV may add some uncertainty to corresponding conclusions.

## Conclusions

In conclusion, our findings define linear PFT changes in a large Italian DMD cohort; strengthen the indication of GC treatment for teenagers and adults living with DMD; identify “distal” DMD mutations, and probably SPP1 and CD40 variants, as risk factors for worse PFT outcomes; and will ultimately be relevant for clinical management, and clinical trial design and interpretation in DMD.

## Acknowledgements

We acknowledge funding from Santhera Pharmaceuticals, which supported the collection and analyses of data from the Italian cohort. We thank Mika Leinonen, MSc, from Santhera Pharmaceuticals, for assistance in the statistical analyses. The CINRG-DNHS was funded by the Department of Education/NIDRR (#H133B031118, #H133B090001); U.S. Department of Defense (#W81XWH-12-1-0417); National Institutes of Health/NIAMS (#R01AR061875); Parent Project Muscular Dystrophy. We are deeply grateful to all participating patients and their families.

# Aim 2 - Genetic modifiers of cardiac function in Duchenne muscular dystrophy

## Introduction

The improvement of the standard of care for DMD patients, and in particular nocturnal ventilation and spinal stabilisation, has improved the life expectancy of DMD patients (Eagle et al., 2002; Klitzner et al., 2005), and at the same time has increased the incidence of dilated cardiomyopathy (DCM), as the probability of being diagnosed with DCM increases with age. It is estimated that 25% of boys have cardiomyopathy at 6 years of age, 59% by 10 years of age and more than 90% of young men over 18 years of age demonstrate evidence of cardiac dysfunction (Nigro et al., 1990).

Considering the increasing impact of DCM on DMD patients, we are interested in studying the influence of known modifiers of disease phenotype on cardiac function. In particular, we are interested in analysing the effect of glucocorticoids treatment, as this is the main treatment for DMD patients but its result on cardiac health is still controversial (Ashwath et al., 2014; Markham et al., 2008; Silversides et al., 2003; Spurney et al., 2014), and the effect of modifier SNPs, especially because it is well known that *in trans* genetic modifiers of DMD act manly on inflammation and fibrosis (i.e. SNPs in *LTBP4*, *SPP1* and *CD40*), both involved in DCM pathogenesis.

## Methods

### *Cohorts*

We collected retrospective left ventricle ejection fraction -henceforth “ejection fraction” (EF), left ventricle end-diastolic volume - henceforth “end-diastolic volume” (EDV), and left ventricle fractional shortening - henceforth “fractional shortening” (FS) data from several Italian Centres, from April 1984 to November 2018, with the aim of describing the effects of GCs treatment, age, modifier SNPs and *DMD* mutations on cardiac performance in DMD patients.

### *Ethics statement*

All participants or their parents/guardians provided informed consent to study procedures, which were carried out in accordance with the Declaration of Helsinki and approved by Ethics Committees/Institutional Review Boards at participating Institutions.

### *Inclusion criteria*

All patients included in this study present *DMD* mutations leading to absent or <3% dystrophin by immunohistochemistry (IHC, except revertant fibres) or immunoblot. Each patient included in this

study has at least one evaluation for at least one of the cardiac outcomes considered in the analysis (EF, EDV and FS).

### *Echocardiographic evaluation*

Echocardiographic studies were performed with Philips SONOS 5500 instruments with a 3 MHz transducer or equivalent instruments. Two-dimensional images and M-mode echocardiograms of atrial and ventricular cavities were obtained in multiple cross-sectional planes, with the transducer in standard positions according to the recommendations of the American Society of Echocardiography (Schiller et al., 1989). Left ventricular ejection fraction (EF) was calculated from two-dimensional images with modified Simpson's formula or area–length method (Schiller et al., 1989).

### *DMD genotype*

Information about pathogenetic *DMD* mutations were collected when available from clinical records or genetic reports. We classified deletions based on amenability to molecular treatments, i.e. skipping of exons 8, 44, 45, 51, and 53 (henceforth: "skip 8", "skip 44", etc.). Nonsense and splice site mutations were considered as separate groups. Moreover, all mutations were subdivided into "proximal", i.e. situated 5' of intron 44, and "distal", i.e. involving intron 44 and/or regions 3' of it. The hypothesis is that distal mutations are predicted to alter the expression of short dystrophin isoforms (Dp140, Dp116, and Dp71), and therefore may be associated with central nervous system involvement that might secondarily affect gross and fine motor performance (Doorenweerd et al., 2017; Felisari et al., 2000; Magri et al., 2011).

### *SNPs genotypes*

When DNA samples were available, patients were genotyped, using TaqMan (Thermo Fisher Scientific) assay, at the known DMD modifier loci located in genes expressed in cardiac tissue: *SPP1* rs28357094 (Pegoraro et al., 2011), *LTBP4* rs10880, rs2303729 and rs1131620 (Flanigan et al., 2013), *CD40* rs1883832 (Bello et al., 2016a), *THBS1* rs2725797 and rs2624259 (Weiss et al., 2018). We also considered the IAAM haplotype made of rs10880, rs2303729 and rs1131620 in *LTBP4* as a modifier. For tests of genotype/phenotype correlation, we used the same inheritance models as in published reports. The genotype of modifier SNPs included in the study respect the Hardy-Weinberg equilibrium.

### *Statistical analysis*

Quantitative variables were summarized as mean ± standard error (SE) and median (range), unless otherwise specified. Generalized Estimating Equations (GEEs) were used to estimate effects of: age; GC treatment (on vs. off at each evaluation); *DMD* mutation (tested separately: each specified mutation group vs. "other" mutations; or "distal" vs. "proximal"); and SNP genotypes (dominant,

recessive, or additive as appropriate). In the GEE analysis covariates are considered as independent one from the others, so rs10880 and IAAM haplotype had been included in separate analyses, as well as *DMD* mutation types and position. Statistical significance was set at p-value <0.01 (Bonferroni correction for 5 genetic loci: *DMD*, *SPP1*, *LTBP4*, *CD40*, *THBS1*). Statistical analyses were performed using R v.3.5.2.

## Results

### Demographics

We collected 1135 EF measurement from 343 DMD patients, 343 SF measurements from 78 patients and 347 EDV values from 142 DMD from the Italian Network. They underwent 4.35 ± 4.62 (± standard deviation) evaluations (maximum 32), with intervals of 0.79 ± 1.33 years, for a follow-up time of 2.67 ±3.79 years (maximum 26.26 years). Population average age at baseline was 14.10 ± 7.06 (maximum 45.09) years and at the end of the study it was 16.71 (maximum 45.09) years old.

### GCs treatment

It was possible to collect complete data about glucocorticoids treatment throughout all the follow-up for 188 out of 360 DMD patients (52.23%). Glucocorticoids coverage of the population is summarised in Table 12.

*Table 12. Distribution by glucocorticoid treatment and demographics of treatment subgroups.*

| Treatment subgroup | n (%) | Mean age in years ± SD | Median age in years (min - max) |
|---|---|---|---|
| Continuously off GCs | 90 (25%) | 18.45±6.39 | 18.80 (4.37 - 33.26) |
| Continuously on GCs | 78 (21.66%) | 12.89±5.01 | 12.25 (3.66 - 28.40) |
| Started GCs during FU | 10 (2.77%) | 9.63±4.24 | 9.17 (3.08 - 20.79) |
| Stopped GCs during FU | 8 (2.22%) | 20.39±2.54 | 20.34 (16.96 - 23.75) |
| Multiple switches | 2 (0.55%) | 7.07±2 | 6.85 (4.65 - 10.62) |
| Unknown or incomplete follow-up data | 172 (47.77%) | 15.91±6.67 | 15.09 (0.76 - 45.09) |
| **Total** | 360 (100%) | 11.16±5.15 | 10.08 (4.17 - 28.57) |

*GCs: glucocorticoids; FU: follow-up; n (%): population numerosity and percentage; SD: standard deviation; min-max: minimum and maximum age for each treatment subgroup.*

### *DMD* mutations

*DMD* mutations were defined in 256 patients (71.11%). In this analysis, we subdivided deletions based on their amenability to molecular treatment. Those that are treatable with antisense oligonucleotide to promote exon skipping are reported as for instance “skip 8”, if exon 8 can be skipped to restore the reading frame (Table 13).

*Table 13. DMD mutations*

| Mutation group | | n (%) | |
|---|---|---|---|
| Deletions | skip 8 | | 3 (1.17%) |
| | skip 44 | | 10 (3.9%) |
| | skip 45 | | 11 (4.30%) |
| | skip 51 | | 23 (8.98%) |
| | skip 53 | | 24 (9.37%) |
| | other | | 115 (44.92%) |
| Duplications | | 22 (8.59%) | |
| Nonsense mutations | | 20 (7.81%) | |
| Small FS mutations | | 8 (3.12%) | |
| Splice site mutations | | 10 (3.9%) | |
| Missense | | 4 (1.56%) | |
| Insertion | | 1 (0.39%) | |
| Other mutations | | 5 (1.95%) | |
| **Total (molecularly defined)** | | 256 (100%) | |
| Proximal mutations (5’ intron 44) | | 99 (36.94%) | |
| Distal mutations (3’ intron 44) | | 157 (59.33%) | |
| **Total (molecularly defined)** | | 256 (100%) | |

*“skip 8”: deletion amenable to treatment by antisense oligonucleotide promoting the skipping of exon 8. Same for other exon numbers; FS: frameshifting.*

## Genetic modifiers genotype

To exclude genotyping errors data had been tested for the Hardy-Weinberg equilibrium (HWE) that was respected for all considered loci (Table 14**Error! Reference source not found.**).

*Table 14. Genetic modifiers genotype*

| SNPs | Obs. major allele (p) frequency | Obs. minor allele (q) frequency | MAF in Europeans (not Finnish) | Obs. heterozygote (2pq) percentage | HWE heterozygote (2pq) percentage |
|---|---|---|---|---|---|
| ***SPP1*** **rs28357094** | 0.79 | 0.26 | 0.24 | 0.32 | 0.34 |
| ***LTBP4*** **rs10880** | 0.64 | 0.36 | 0.47 | 0.43 | 0.47 |
| ***CD40*** **rs1883832** | 0.75 | 0.25 | 0.26 | 0.38 | 0.37 |
| ***THBS1*** **rs2725797** | 0.82 | 0.28 | 0.16 | 0.29 | 0.32 |

*Obs. = observed; MAF= minor allele frequency; Hardy-Weinberg equilibrium (HWE): p2+2pq+q2=1. p = frequency of the dominant allele; q= frequency of the recessive allele; p2 =frequency of individuals with the homozygous dominant genotype; 2pq = frequency of individuals with the heterozygous genotype; q2= frequency of individuals with the homozygous recessive genotype.*

## Effects of GCs and age

We used the generalised estimated equations model to evaluate the effect of age and GCs treatment on heart function evaluated as EF, EDV and FS, results are detailed in Table 15Table 15. We found that age impacts only EF, with a yearly decline of 0.85% ± 0.16 (p-value <0.0001). It was also possible to detect a slight protective effect of GCs treatment on EF (+0.11% ± 1.47 p-value <0.001) (Figure 27). The GEE model did not find any effect of GCs treatment nor age on FS values, even if it is noticeable in Figure 27 C that treated and untreated patients have close baseline FS values, but fractional shortening decreases more with age in untreated patients. We detected a similar effect of age and glucocorticoids on EDV values, in particular the model predicted that GCs treatment has a more evident impact on older patients (Figure 27 B).

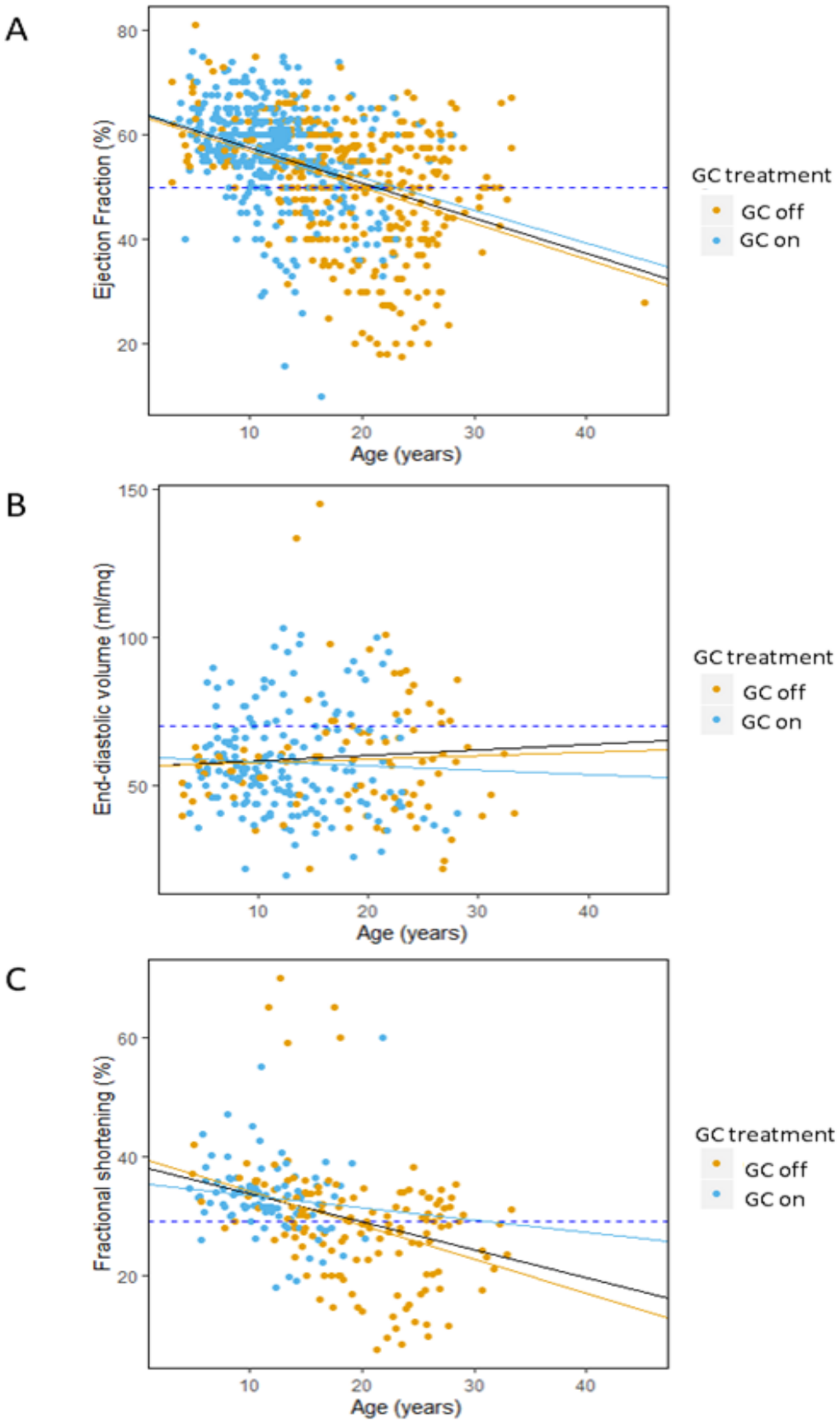


*Figure 27. Scatter plots of ejection fraction (%) (A), end-diastolic volume (ml/m$^2$) (B) and fractional shortening (%) (C) by age, grouped by GC treatment at evaluation time. Pathological thresholds are marked by dashed lines. Black regression lines describe the whole population.*

### *DMD* mutation effects

The present study allowed to detect the effect of different mutations on variable cardiac parameters (Table 15). Mutations amenable to skip exon 44 have a significant effect (p-value < 0.01) on the decrease of EDV (-15.66 ml/$m^2$ ± 5.07). A significant effect on the opposite direction (+16.77 ml/$m^2$ ± 4.51, p-value < 0.01) had been observed also from skip 45 mutations (unfortunately were not enough EDV data of patients carrying this mutation to derive a regression line). Skip 53 mutation group is associated with higher EDV than other mutations (Figure 28 B), even if in the GEE analysis did not reach the statistical significance. *DMD* mutations did not have effect on EF and FS in our cohort; nevertheless nonsense mutations have a nominally significant (p-value= 0.04) protective effect on EF (+4.29% ± 2.08) (Figure 28 A), and skip 51 is detrimental for fractional shortening (-3.39% ± 3.48, p-value<0.001) (Figure 28 C).

Finally, we investigated if the position of *DMD* mutations on the gene sequence has an effect on cardiac phenotype. Stratifying DMD mutations in proximal and distal no effect on the studied outcome measures were detected (Table 15).

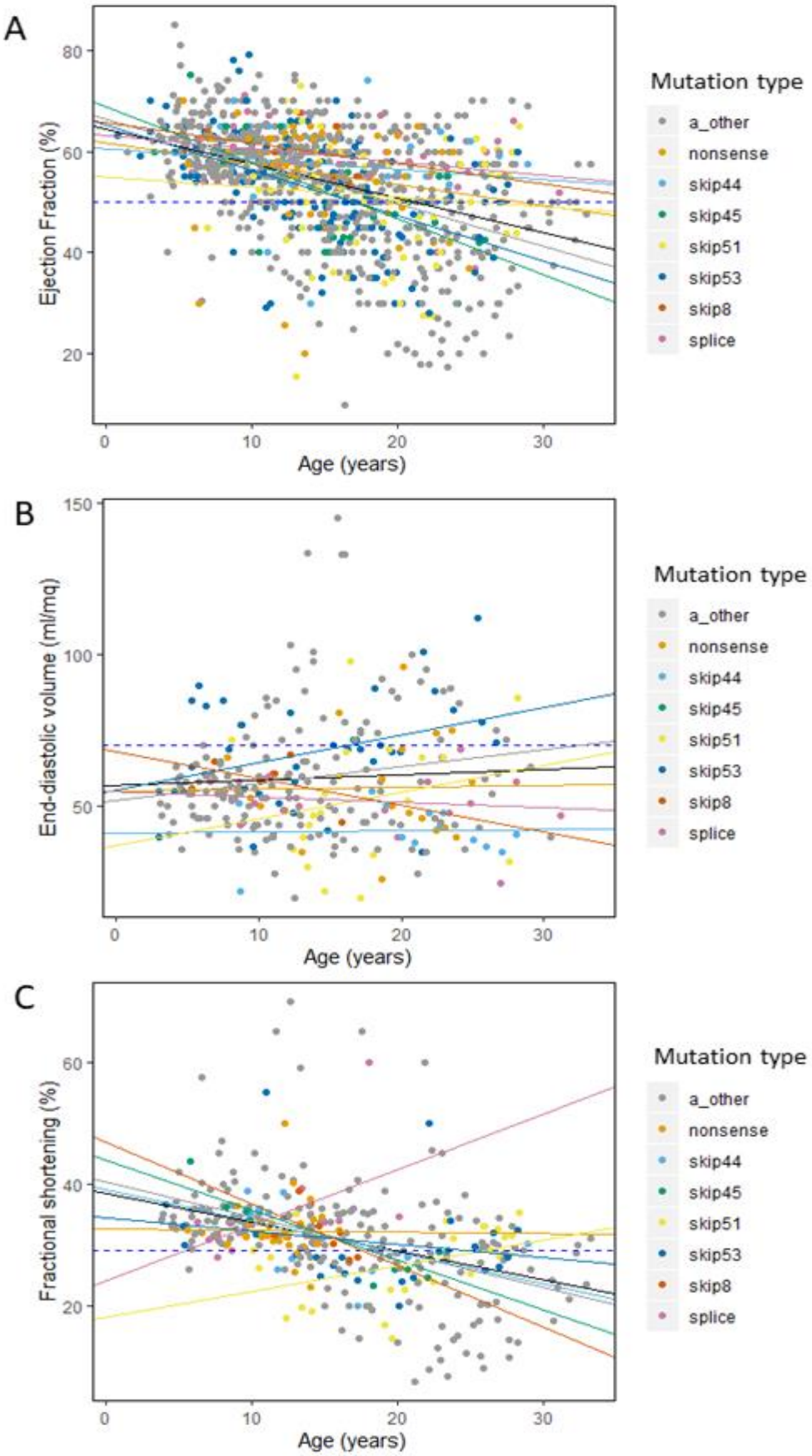


*Figure 28. Scatter plots of ejection fraction (%) (A), end diastolic volume (ml/m$^2$) (B), fractional shortening (%) (C) by age, grouped by mutation type. Pathological thresholds are marked by blue dashed line. Black regression lines describe the whole population.*

## Modifier SNPs effects

GEE analysis found a very strong effect of rs10880 in *LTBP4*, and in particular of the minor haplotype IAAM, on EDV (-9.22 ml/m$^2$ ± 3.18, p<0.01) (Figure 32 A and B) and EF (+4.48% ± 1.6, p<0.01) (Figure 29 A and B) values. No effect of *LTBPP4* SNPs had been detected for FS, but the analysis pointed out a nominally significant protective effect of rs1883832 in *CD40* (+3.97 ± 1.98, p-value = 0.04) (Figure 33 B) on this parameter. The effect of all modifier SNPs is described in Figure 30, Figure 31 and Figure 33.

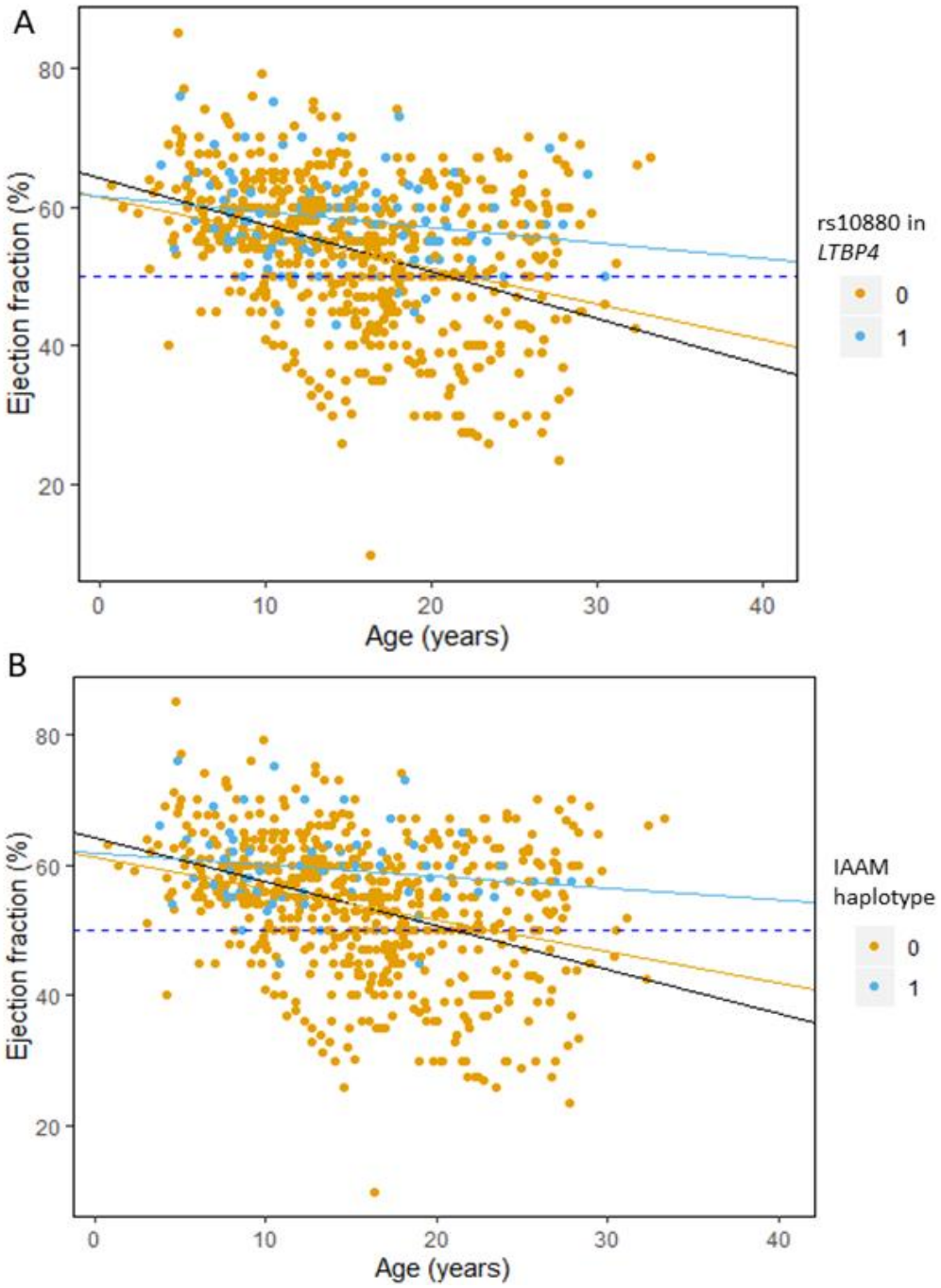


*Figure 29. Scatter plots of ejection fraction (%) by age, grouped by modifier SNP rs10880 in LTBP4 (A) and IAAM haplotype (B) of the same SNP. Ejection fraction lower than 50% is considered pathological, threshold is marked by blue dashed line. The black regression lines describe the whole population.*

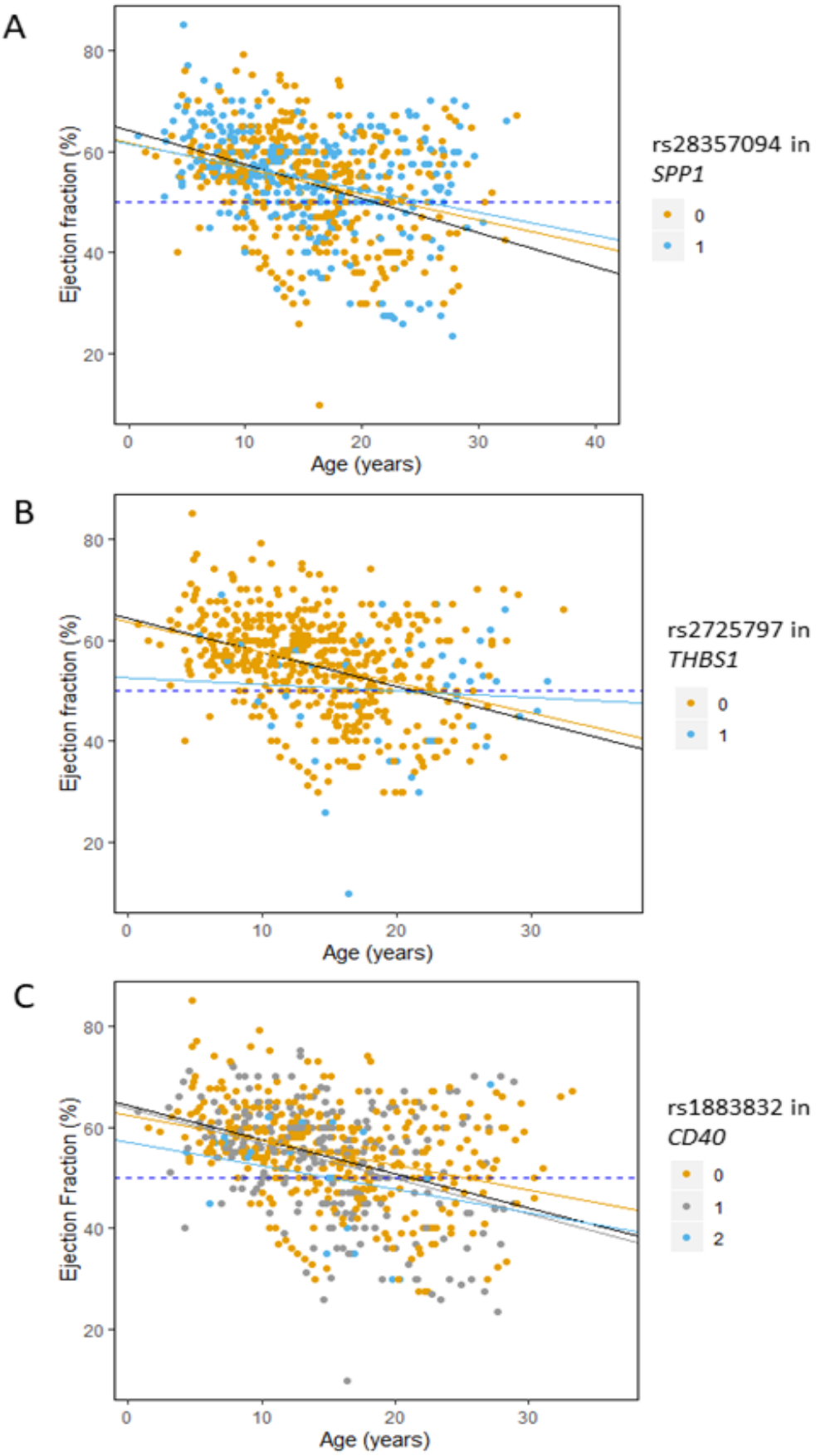


*Figure 30. Scatter plots of ejection fraction (%) by age, grouped by modifier SNPs: rs28357094 in SPP1 (A); rs1883832 in CD40 (B); rs2725797 in THBS1 (C). Ejection fraction lower than 50% is considered pathological, threshold is marked by blue dashed line. The black regression lines describe the whole population.*

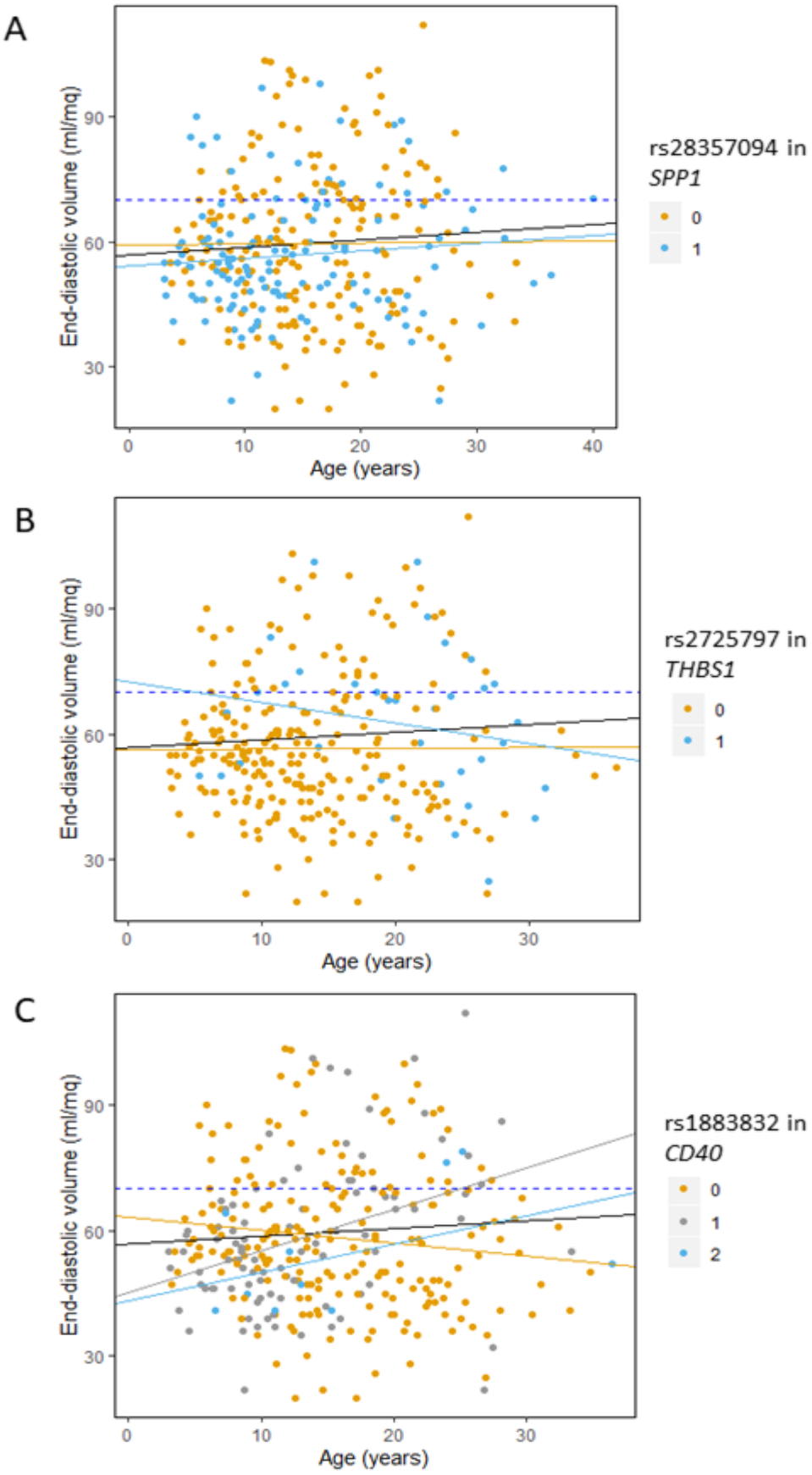


*Figure 31. Scatter plots of end-diastolic volume (ml/m²) by age, grouped by modifier SNPs: rs28357094 in SPP1 (A); rs1883832 in CD40 (B); rs2725797 in THBS1 (C). End-diastolic volume over 70 ml/m² is considered pathological, threshold is marked by blue dashed line. The black regression lines describe the whole population.*

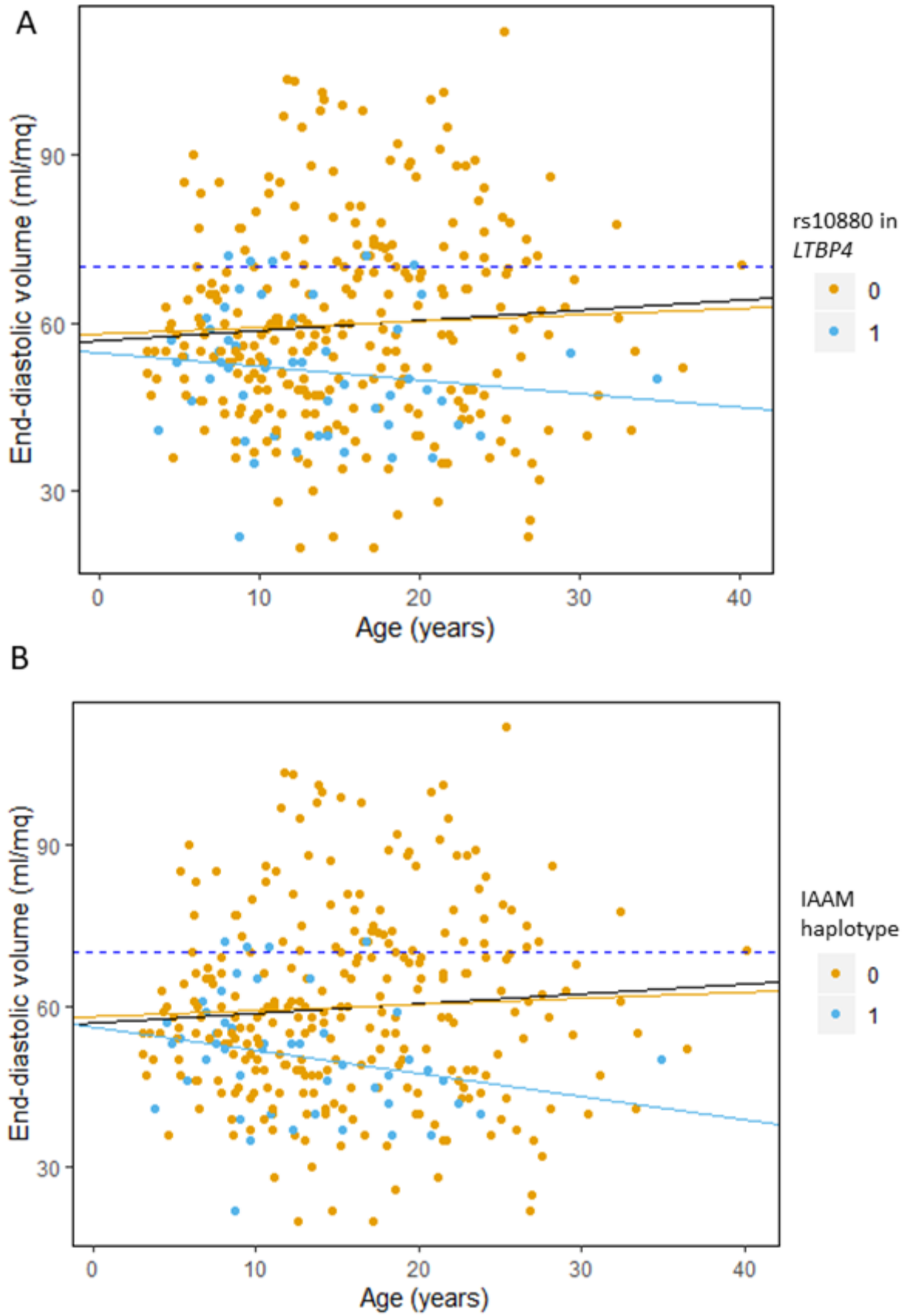


*Figure 32. Scatter plots of EDV (ml/m2) by age, grouped by modifier SNP rs10880 in LTBP4 (A) and IAAM haplotype (B) of the same SNP. End-diastolic volume over 70 ml/m² is considered pathological, threshold is marked by blue dashed line. The black regression lines describe the whole population.*

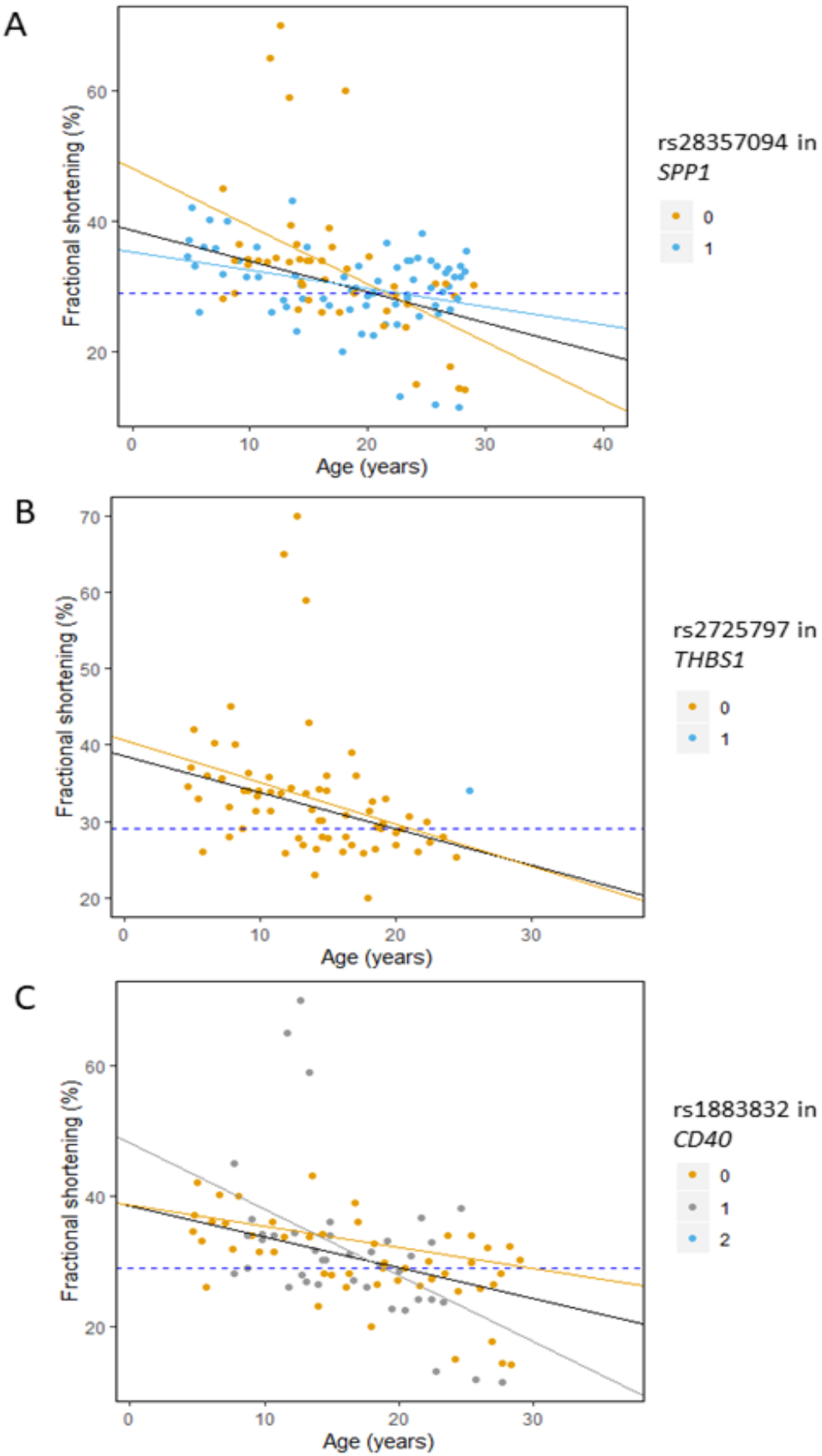


*Figure 33. Scatter plots of fractional shortening (%) by age, grouped by modifier SNPs: rs28357094 in SPP1 (A); rs1883832 in CD40 (B); rs2725797 in THBS1 (C). Fractional shortening below 29% is considered pathological, threshold is marked by blue dashed line. The black regression lines describe the whole population.*

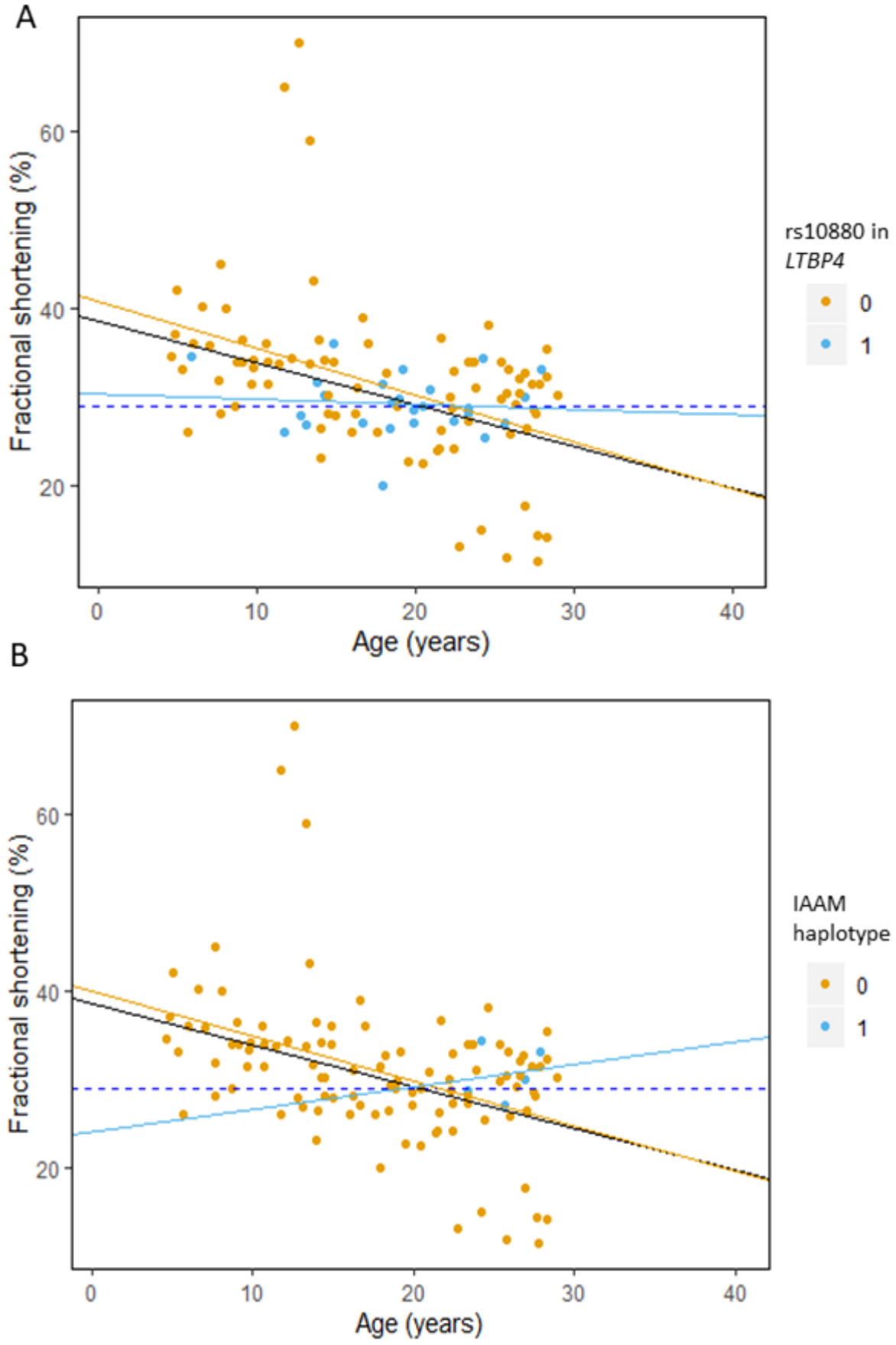


*Figure 34. Scatter plots of fractional shortening (%) by age, grouped by modifier SNP rs10880 in LTBP4 (A) and IAAM haplotype (B) of the same SNP. Fractional shortening below 29% is considered pathological, threshold is marked by blue dashed line. The black regression lines describe the whole population.*

*Table 15. Coefficients of Generalized Estimating Equation (GEE) analyses*

| | | EF (%) | | EDV (ml/m²) | | FS (%) | |
|---|---|---|---|---|---|---|---|
| **Coefficient** | | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** | **Estimate ± SE** | **p-value** |
| **Intercept** | | 66.61 ± 3.09 | **< 0.0001** | 44.42 ± 5.66 | **< 0.0001** | 36.11 ± 4.87 | **< 0.0001** |
| **Age (per-year decrease)** | | -0.85 ± 0.16 ↓↓ | **< 0.0001** | 0.55± 0.39 | n.s. | -0.44± 0.24 | n.s. |
| **GC treatment** | | 0.11 ± 1.47 ↑↑ | **< 0.001** | 2.31 ± 2.31 | n.s. | -1.38 ± 2.42 | n.s. |
| **Mutation 5' exon 44** | | 0.53 ± 1.45 | n.s. | 3.68 ± 3.54 | n.s. | 5.26 ± 2.93 | n.s. |
| **Mutation type** | **Nonsense** | 4.29 ± 2.08 ↑ | **0.04** | 0.68 ± 6.10 | n.s. | 1.26 ± 2.06 | n.s. |
| | **skip 44** | 0.32 ± 4.39 | n.s. | -15.66 ± 5.07 ↓↓ | **<0.01** | 1.26 ± 2.06 | n.s. |
| | **skip 45** | -3.63 ± 3.96 | n.s. | 16.77 ± 4.51 ↑↑ | **<0.001** | -0.31 ± 2.97 | n.s. |
| | **skip 51** | 1.65 ± 2.85 | n.s. | -4.26 ± 6.53 | n.s. | -3.39± 3.48 ↓↓ | **<0.001** |
| | **skip 53** | -0.45 ± 2.40 | n.s. | 9.05 ± 5.20 | n.s. | -0.56 ± 1.90 | n.s. |
| | **skip 8** | 6.33± 3.76 | n.s. | 4.48 ± 5.43 | n.s. | -0.44 ± 1.52 | n.s. |
| | **splice** | 3.52 ± 2.96 | n.s. | -0.57 ± 4.62 | n.s. | 0.52 ± 4.10 | n.s. |
| **SNP modifiers** | **rs28357094 dom** | -0.36 ± 1.44 | n.s. | 5.04± 3.17 | n.s. | -2.02± 2.93 | n.s. |
| | **rs10880 rec** | 4.48 ± 1.60 ↑↑ | **<0.01** | -9.22 ± 3.18 ↓↓ | **<0.01** | 0.65 ± 3.34 | n.s. |
| | **IAAM haplotype** | 4.04 ± 1.80 ↑ | **0.02** | -10.57 ± 3.01 ↓↓ | **<0.001** | | |
| | **rs1883832 add** | -1.93 ± 1.27 | n.s. | 1.57 ± 3.18 | n.s. | 3.97 ± 1.98 ↑ | **0.04** |
| | **rs2725797 rec** | 0.07 ± 2.92 | n.s. | | | 5.80 ± 4. | n.s. |

*EF: ejection fraction; EDV: end diastolic volume; FS: fractional shortening; SE: Standard Error; GC: glucocorticoid corticosteroids; Skip 8: mutations amenable to treatment with skipping of exon 8 (same for other exon numbers); n.s.: not significant; dom: dominant inheritance model; add: additive inheritance model; rec: recessive inheritance model. Nominally significant effects have been marked with arrows, upward for positive effects and downward for negative. Double arrows indicate "strong". p-values <0.06 (nominally significant) or lower are highlighted in **bold**. Barred boxes correspond to covariates non included in the GEE analysis.*

## Discussion

The present study is a retrospective evaluation of the effect of environmental, *cis*-acting and *trans*-acting modifiers on cardiac function of Duchenne muscular dystrophy, using a Generalised Estimating Equations model.

We studied GCs impact on cardiac health with the aim to shed a light on the controversy about they to be protective or detrimental for DCM progression (Ashwath et al., 2014; Markham et al., 2008; Silversides et al., 2003; Spurney et al., 2014). We found that the treatment is slightly protective when we considered ejection fraction as indicator of cardiac function. We do not find the same result looking at end-diastolic volume or fractional shortening, this is particularly surprisingly considering the tight connection between EF and EDV. Our hypothesis is that this discrepancy is due to the disproportion between the numerosity of EF (1135) and EDV and FS (347 and 343 respectively) evaluations in our database. This is also the reason why we did not use the age of onset of DCM in our population as outcome parameter in the analysis, as it had been done in other studies aiming to evaluate modifiers of cardiac function in Duchenne muscular dystrophy patients (Barp et al., 2015; Jefferies et al., 2005; Van Dorn et al., 2018). In fact, DCM onset is usually defined as the age at the first echocardiographic finding of LV end-diastolic volume >70 ml/$m^2$, and/or LV-EF <50%, and in our population very few patients have both data reported for the same visit.

Finally, to evaluate the lack of a strong effect of GCs treatment on cardiac parameters, we have to consider both potential disease-changing effects of cardio-protective treatments followed by patients for which we do not have records, and the not randomisation of GCs assumption. Moreover, GCs findings. The lack of randomisation is one of the main concerns in retrospective analyses, and in our study it heavily impacts the result, as older patients are more likely to be GCs off that younger, introducing a bias in the analysis (Table 12).

We also considered the role of *cis*-acting factors, i.e. mutations in *DMD* gene, on cardiac parameters. In the past lots of effort had been done to determine if mutations affecting different protein's domain have different effect of DMD cardiac phenotype, with contrasting results (Jefferies et al., 2005; Van Dorn et al., 2018). For this reason, and to increase the relevance of our analysis for future clinical trials, in this study we decided to focus only on specific mutation and grouping them base on their amenability to exon skipping treatment. It is know from literature that skip 44 mutations are associated with a later loss of ambulation in DMD patients (Wang et al., 2018), we confirmed the protective role of these mutations also on cardiac phenotype as it is associated with lower end-diastolic volume. The present study also corroborates the detrimental effect of skip 51 mutations known from literature (Wang et al., 2018) and previously confirmed by our group also for respiratory

function in DMD patients (refer to “Aim 2 - Genetic modifiers of performance of the upper limbs in Duchenne muscular dystrophy patients”).

With concern to *trans*-acting factor, it is known that IAAM haplotype in *LTBP4* is more frequent in patients without left ventricular disfunctions, but this data never reached the statistical significance (Barp et al., 2015; Van Dorn et al., 2018). In the present study we were able to demonstrate the impact of IAAM haplotype with all the outcome measures tested.

From a molecular point of view the protective role of this modifier can be explained with the reduced TGF-β signalling that characterises this haplotype as demonstrated by Flanigan et al. (Flanigan et al., 2013) (refer to “*LTBP4* (Latent Transforming Growth Factor β Binding Protein 4)” for more details).

The present study also allowed to identify a nominally significant protective effect of *CD40* rs1883832 in never reported before. This data will need further investigation but is promising considering the role of the modifier SNP in inflammation processes (check “*CD40 (Cluster of Differentiation 40)*, also known as *TNFRSF5* (Tumour Necrosis Factor Receptor SuperFamily Member 5)”).

# Side project – Three-dimensional *in vitro* modelling of neuromuscular diseases

## Introduction

I spent the vast majority of the last year of my PhD in Penney Gilbert's Laboratory at the University of Toronto. Here I used the MyoTACTIC, a three-dimensional (3D) *in vitro* platform, here developed, to model Duchenne muscular dystrophy and central core disease (CCD).

Animal models have a pivotal role in the study of human neuromuscular disorders, , but their use is limited by ethical and economic considerations (Pampaloni et al., 2007). By contrast, two-dimensional (2D) human cell culture systems are cheap and easy to work with, and minimize ethical concerns, but lack tissue-specific architecture and the mechanical and biochemical signaling that characterize adult human skeletal muscle (Pampaloni et al., 2007). Thus, several laboratories are implementing 3D human cell culture methodologies to boost myofiber structural maturation *in vitro* (Bachmann et al., 2019).

Generally, 3D skeletal muscle cell culture involves the encapsulation of muscle progenitor cells within a biomaterial and then deposit these into a culture well containing two attachment points, that mimic the tendons, to establish uniaxial tension and drive the self-organization of aligned, multinucleated myofibers in the 3D tissue construct (Figure 35 C, D). Unlike 2D culture, where cells bind a substrate only on one side and maturation is affected by aberrant signalling from the cell surface to the nucleus (Baker and Chen, 2012), a 3D matrix allows the cells to spatially organize and assemble into architectures closer to the native physiological conditions (Smith et al., 2016), mimicking native tissue structure and function, thereby providing a promising system for disease modelling, drug discovery or pre-clinical validation, and toxicity testing. Widespread adoption of this research approach is hindered by the lack of an easy-to-use platform that is simple to fabricate and yields arrays of human skeletal muscle micro-tissues (hMMTs) in culture with reproducible physiological responses that can be assayed non-invasively (reviewed in Fusto et al., 2019 pre-print).

Penney Gilbert's group developed a polydimethylsiloxane 96-well platform (called MyoTACTIC), that enables bulk production of 3D hMMTs. After differentiation hMMTs show mature contractile apparatus and are responsive to electrical and chemical stimulation that can be performed in a non-invasive way. MyoTACTIC also allows non-invasive calcium transient and contractile force measurements on hMMTs (Afshar et al., 2019) (Figure 35).

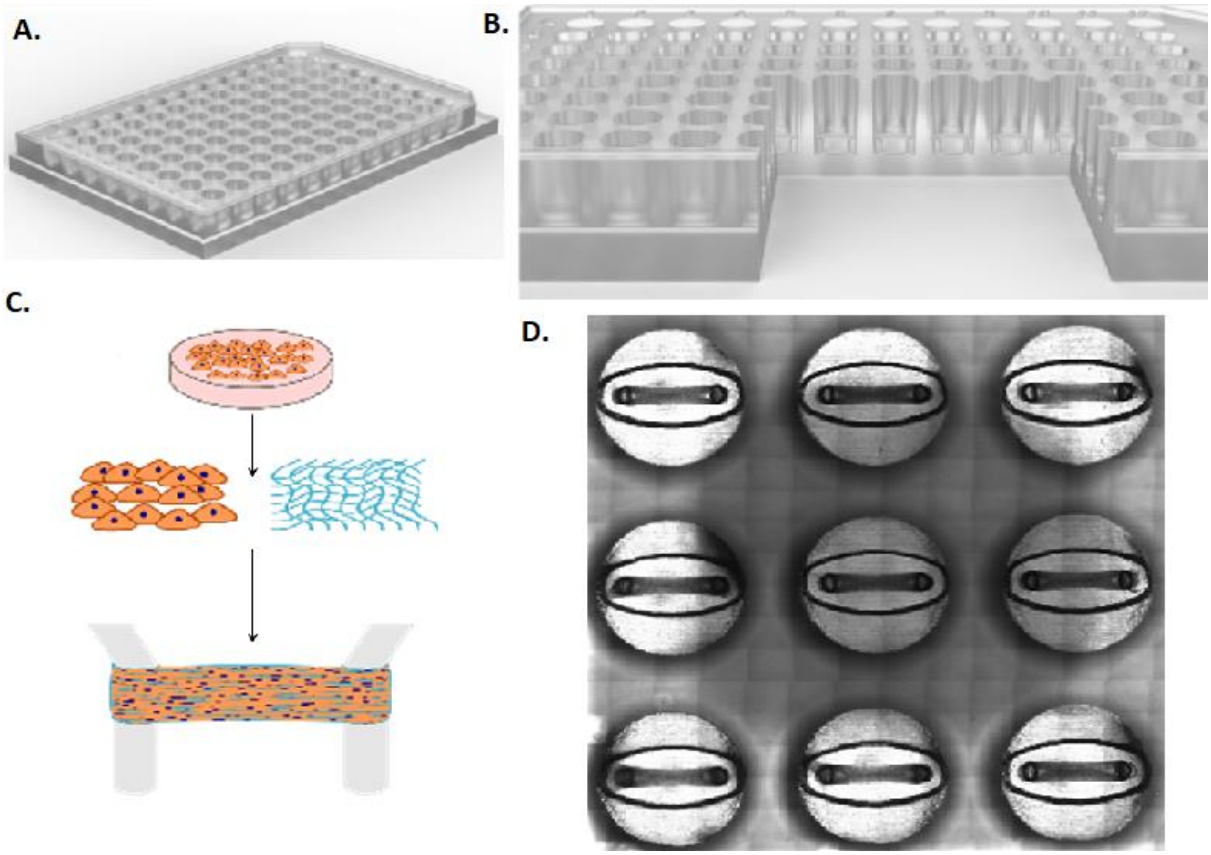


*Figure 35. MyoTACTIC. A, B) Computer generated 3D images of MyoTACTIC 96-well plate design and (B) a cross-section of wells indicating the location of the micro-posts. C) Schematic overview of generation of hMMTs in MyoTACTIC.D) Stitched phase-contrast image of 9 wells of MyoTACTIC containing remodelled hMMTs 10 days post seeding. Scale bar 5 mm. Adapted from Afshar et al., 2019.*

## *In* vitro three-dimensional modelling of Duchenne muscular dystrophy

*In vitro* study of Duchenne muscular dystrophy with 2D muscle cultures cannot fully recapitulate the complex nature of the disease, as they are lacking fully differentiated myofibers. For this reason, we used immortalized human DMD and healthy myoblast lines to derive hMMTs. Cell lines had been immortalized through lentiviral transfection with hTERT and cdk4 (Mamchaoui et al., 2011).

As DMD muscle biopsies are challenging to obtain and their muscle progenitors are short lived, these immortalized myogenic progenitors are a promising cell source for DMD studies. We optimized cell-densities to seed immortalized myoblasts in a fibrin-based hydrogel, and differentiation conditions which permit hMMT self-organization over a period of 10 days. DMD and healthy hMMTs are comprised of aligned myotubes expressing mature sarcomere proteins. Compared to healthy hMMTs, DMD hMMTs exhibit alterations in tissue remodelling process, in the calcium handling in response to acetylcholine stimulation, and membrane stability. We also find that myotubes in DMD hMMTs are ~41% smaller in width in comparison with their healthy counterparts.

Our data indicate that hMMTs produced using immortalized healthy and DMD myoblasts provide a reliable testing platform for therapeutic approaches by allowing the study of biological mechanisms associated with DMD, and serving as a promising tool for DMD drug discovery.

## *In* vitro three-dimensional modelling of central core disease

I used the same platform to model central core disease, a congenital skeletal muscle myopathy caused by mutations in *RYR1* gene. The disease is histologically characterized by the presence of "cores", areas lacking in mitochondria that show abnormal oxidative enzymatic activity and sarcomere's disruption, in the centre of a variable proportion of type I muscle fibres (Jungbluth et al., 2011).

The study of CCD using traditional *in vitro* models has enabled the clarification of the two pathogenic mechanisms (leaky channel and excitation-contraction uncoupling). However, 2D models cannot be used to understand how pathogenic cores are formed, as they do not appear in 2D.

Using MyoTACTIC, we derive hMMTs from a primary CCD line. Oxidative stainings (COX, SDH, NADH) of transverse cryosections obtained from these hMMTs showed the presence of cores-like areas in some fibres.

Moreover, CCD hMMTs developed more spontaneous contractile activity than healty hMMTs, suggesting altered calcium equilibrium. These characteristics recapitulate the disease phenotype and provide evidence that 3D models are superior to 2D for studying CCD.

Due to the limitations of primary myoblast lines (i.e. genetic background, senescence) we moved towards a gene editing strategy (CRISPR-Cas9) to develop isogenic clones of immortalised myoblasts containing patient-specific mutations.

Moreover, we transduced the immortalised lines with Channel Rhodopsin and the calcium reporter GCaMP6 in order to quantitatively assess altered calcium handling and muscle weakness in CCD.

## Conclusions

Even if the both DMD and CCD modelling in MyoTACTIC is still in a preliminary stage, we think that the platform and the usage of immortalised cell lines are extremely promising and may clarify several unclear aspects of neuromuscular disorders, for instance it may help to understand cores development in CCD.

# Conclusion

Duchenne muscular dystrophy is a severe X-linked disease, that affects males since very young age. The disease is caused by out-of-frame mutations in the *DMD* gene, that result in the complete, or almost complete (<3%) lack of dystrophin in skeletal muscle fibres of affected individuals. This molecular defect causes muscle damage upon contraction leading to fibro-fatty substitution of the tissue.

Even if from a molecular point of view all patients are equal, it is possible to observe different disease's progression rate (i.e. LoA age, cardiomyopathy onset, etc) among DMD patients. This variability is due to environmental and genetic factors, the impact of which had been widely studied used age of loss of ambulation as outcome variable (Bello et al., 2015b; Bello et al., 2016a; Bello et al., 2016b; Dwianingsih et al., 2014; Flanigan et al., 2013; Hogarth et al., 2017; Hufton and Roper, 2017; Pegoraro et al., 2011; Weiss et al., 2018).

The main aim of this thesis was to explore the known sources of variability in DMD, focusing on less studied disease's aspects, such as performance of upper limbs, respiratory function and cardiomyopathy. The reason why we studied these outcomes is that with the increasing number of clinical trials addressed to DMD patients, there is a need of information on sources of variability inside the study population for a better trials' design.

Our analyses allowed to determine rate of progression of respiratory and cardiac function in DMD and confirm the effect of known modifiers on some of the outcome measures considered. We showed a protective effect of *LTBP4* rs10880 on cardiac function and a negative effect of the dominant G genotype at rs28357094 in the *SPP1* promoter on FVC and a negative effect of the additive T genotype at rs1883832 in the *CD40* 5' untranslated region (UTR) on PEF. We finally proved that GCs treatment is beneficial also in non-ambulatory patients and may improve respiratory function.

# Bibliography


**Aartsma-Rus, A. and Krieg, A. M.** (2017). FDA Approves Eteplirsen for Duchenne Muscular Dystrophy: The Next Chapter in the Eteplirsen Saga. *Nucleic Acid Ther.* **27**, 1–3.

**Afshar, M. E., Abraha, H. Y., Bakooshli, M. A., Davoudi, S., Thavandiran, N., Tung, K., Ahn, H., Ginsberg, H., Zandstra, P. W. and Gilbert, P. M.** (2019). A 96-well culture platform enables longitudinal analyses of engineered human skeletal muscle microtissue strength. *bioRxiv* 562819.

**Allen, R. E., Sheehan, S. M., Taylor, R.G., Kendall, T. L. and Rice, G. .** (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J. Cell. Physiol.* 307–312.

**Angelini, C., Pegoraro, E., Turella, E., Intino, M. T., Pini, A. and Costa, C.** (1994). Deflazacort in Duchenne dystrophy: Study of long-term effect. *Muscle Nerve* **17**, 386–391.

**Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R. K. and Chazaud, B.** (2007). Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **204**, 1057 LP – 1069.

**Ashwath, M. L., Jacobs, I. B., Crowe, C. A., Ashwath, R. C., Super, D. M. and Bahler, R. C.** (2014). Left ventricular dysfunction in Duchenne muscular dystrophy and genotype. *Am. J. Cardiol.* **114**, 284–289.

**Bachmann, C., Noreen, F., Voermans, N. C., Schär, P. L., Vissing, J., Fock, J. M., Bulk, S., Kusters, B., Moore, S. A., Beggs, A. H., et al.** (2019). Aberrant regulation of epigenetic modifiers contributes to the pathogenesis in patients with selenoprotein N - related myopathies. *Hum. Mutat.* humu.23745.

**Bakay, M., Zhao, P., Chen, J. and Hoffman, E. P.** (2002). A web-accessible complete transcriptome of normal human and DMD muscle. *Neuromuscul. Disord.* **12**, S125–S141.

**Baker, B. M. and Chen, C. S.** (2012). Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *J. Cell Sci.* **125**, 3015–3024.

**Barbosa-Souza, V., Contin, D. K., Filho, W. B., de Araújo, A. L., Irazusta, S. P. and da Cruz-Höfling, M. A.** (2011). Osteopontin, a chemotactic protein with cytokine-like properties, is up-regulated in muscle injury caused by Bothrops lanceolatus (fer-de-lance) snake venom. *Toxicon* **58**, 398–409.

**Barfield, W. L., Uaesoontrachoon, K., Wu, C. S., Lin, S., Chen, Y., Wang, P. C., Kanaan, Y., Bond, V. and Hoffman, E. P.** (2014). Eccentric muscle challenge shows osteopontin polymorphism modulation of muscle damage. *Hum. Mol. Genet.* **23**, 4043–4050.

**Barp, A., Bello, L., Politano, L., Melacini, P., Calore, C., Polo, A., Vianello, S., Sorarù, G., Semplicini, C., Pantic, B., et al.** (2015). Genetic modifiers of duchenne muscular dystrophy and dilated cardiomyopathy. *PLoS One* **10**, 1–14.

**Beggs, A. H., Byers, T. J., Knoll, J. H., Boyce, F. M., Bruns, G. A. and Kunkel, L. M.** (1992). Cloning and

characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. *J. Biol. Chem.* **267**, 9281–9288.

**Belles-Isles, M., Roy, R., Dansereau, G., Goulet, M., Roy, B., Bouchard, J. P. and Tremblay, J. P.** (1993). Rapid selection of donor myoblast clones for muscular dystrophy therapy using cell surface expression of NCAM. *Eur. J. Histochem.* **37**, 375–380.

**Bello, L. and Pegoraro, E.** (2016). Genetic diagnosis as a tool for personalized treatment of Duchenne muscular dystrophy. *Acta Myol. myopathies cardiomyopathies Off. J. Mediterr. Soc. Myol.* **35**, 122–127.

**Bello and Pegoraro** (2019). The "Usual Suspects": Genes for Inflammation, Fibrosis, Regeneration, and Muscle Strength Modify Duchenne Muscular Dystrophy. *J. Clin. Med.* **8**, 649.

**Bello, L., Piva, L., Barp, A., Taglia, A., Picillo, E., Vasco, G., Pane, M., Previtali, S. C., Torrente, Y., Gazzerro, E., et al.** (2012). Importance of SPP1 genotype as a covariate in clinical trials in Duchenne muscular dystrophy. *Neurology* **79**, 159–162.

**Bello, L., Kesari, A., Gordish-Dressman, H., Cnaan, A., Morgenroth, L. P., Punetha, J., Duong, T., Henricson, E. K., Pegoraro, E., McDonald, C. M., et al.** (2015a). Genetic modifiers of ambulation in the cooperative international Neuromuscular research group Duchenne natural history study. *Ann. Neurol.* **77**, 684–696.

**Bello, L., Gordish-Dressman, H., Morgenroth, L. P., Henricson, E. K., Duong, T., Hoffman, E. P., Cnaan, A. and McDonald, C. M.** (2015b). Prednisone/prednisolone and deflazacort regimens in the CINRG Duchenne Natural History Study. *Neurology* **85**, 1048 LP – 1055.

**Bello, L., Flanigan, K. M., Weiss, R., United Dystrophinopathy Project, Spitali, P., Aartsma-Rus, A., Muntoni, F., Mercuri, E., Tuffery-Giraud, S., Claustres, M., et al.** (2016a). Association Study of Exon Variants in the NF-κB and TGFβ Pathways Identifies CD40 as a Modifier of Duchenne Muscular Dystrophy. *Am. J. Hum. Genet.* **99**, 1163–1171.

**Bello, L., Morgenroth, L. P., Gordish-Dressman, H., Hoffman, E. P., McDonald, C. M. and Cirak, S.** (2016b). DMD genotypes and loss of ambulation in the CINRG Duchenne Natural History Study. *Neurology* **87**, 401 LP – 409.

**Biggar, W. D., Gingras, M., Fehlings, D. L., Harris, V. A. and Steele, C. A.** (2001). Deflazacort treatment of Duchenne muscular dystrophy. *J. Pediatr.* **138**, 45–50.

**Biressi, S., Miyabara, E. H., Gopinath, S. D., M. Carlig, P. M. and Rando, T. A.** (2014). A Wnt-TGFβ2 axis induces a fibrogenic program in muscle stem cells from dystrophic mice. *Sci. Transl. Med.* **6**, 267ra176 LP-267ra176.

**Birnkrant, D. J., Bushby, K., Bann, C. M., Alman, B. A., Apkon, S. D., Blackwell, A., Case, L. E., Cripe, L., Hadjiyannakis, S., Olson, A. K., et al.** (2018a). Diagnosis and management of Duchenne

muscular dystrophy, part 2: respiratory, cardiac, bone health, and orthopaedic management. *Lancet Neurol.* **17**, 347–361.

**Birnkrant, D. J., Bushby, K., Bann, C. M., Apkon, S. D., Blackwell, A., Brumbaugh, D., Case, L. E., Clemens, P. R., Hadjiyannakis, S., Pandya, S., et al.** (2018b). Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and neuromuscular, rehabilitation, endocrine, and gastrointestinal and nutritional management. *Lancet Neurol.* **17**, 251–267.

**Blanchard, A., Ohanian, V. and Critchley, D.** (1989). The structure and function of α-actinin. *J. Muscle Res. Cell Motil.* **10**, 280–289.

**Bonifati, M. D., Ruzza, G., Bonometto, P., Berardinelli, A., Gorni, K., Orcesi, S., Lanzi, G. and Angelini, C.** (2000). A multicenter, double-blind, randomized trial of deflazacort versus prednisone in duchenne muscular dystrophy. *Muscle and Nerve* **23**, 1344–1347.

**Bowen, T., Jenkins, R. H. and Fraser, D. J.** (2013). MicroRNAs, transforming growth factor beta-1, and tissue fibrosis. *J. Pathol.* **229**, 274–285.

**Brogna, C., Coratti, G., Pane, M., Ricotti, V., Messina, S., D'Amico, A., Bruno, C., Vita, G., Berardinelli, A., Mazzone, E., et al.** (2019). Long-term natural history data in Duchenne muscular dystrophy ambulant patients with mutations amenable to skip exons 44, 45, 51 and 53. *PLoS One* **14**, e0218683.

**Capote, J., Kramerova, I., Martinez, L., Vetrone, S., Barton, E. R., Sweeney, H. L., Miceli, M. C. and Spencer, M. J.** (2016). Osteopontin ablation ameliorates muscular dystrophy by shifting macrophages to a pro-regenerative phenotype. *J. Cell Biol.* **213**, 275 LP – 288.

**Cayer, D. M., Nazor, K. L. and Schork, N. J.** (2016). Mission critical: the need for proteomics in the era of next-generation sequencing and precision medicine. *Hum. Mol. Genet.* **25**, R182–R189.

**Ceco, E., Bogdanovich, S., Gardner, B., Miller, T., DeJesus, A., Earley, J. U., Hadhazy, M., Smith, L. R., Barton, E. R., Molkentin, J. D., et al.** (2014). Targeting latent TGFβ release in muscular dystrophy. *Sci. Transl. Med.* **6**, 259ra144 LP-259ra144.

**Chang, N. C. and Rudnicki, M. A.** (2014). Chapter Six - Satellite Cells: The Architects of Skeletal Muscle. In *Stem Cells in Development and Disease* (ed. Rendl, M. B. T.-C. T. in D. B.), pp. 161–181. Academic Press.

**Chen, G., Birnbaum, R. S., Yablonka-Reuveni, Z. and Quinn, L.** (1994). Separation of mouse crushed muscle extract into distinct mitogenic activities by heparin affinity chromatography. *J. Cell Sci.* 563–572.

**Chen, Y., Ali, T., Todorovic, V., O'Leary, J. M., Kristina Downing, A. and Rifkin, D. B.** (2005a). Amino Acid Requirements for Formation of the TGF-β-Latent TGF-β Binding Protein Complexes. *J. Mol. Biol.* **345**, 175–186.

**Chen, Y., Nagaraju, K., Bakay, M., McIntyre, O., Rawat, R., Shi, R. and Hoffman, E. P.** (2005b). Early onset of inflammation and later involvement of TGFβ in Duchenne muscular dystrophy. *Neurology* **65**, 826 LP – 834.

**Chen, B., Cole, J. W. and Grond-Ginsbach, C.** (2017). Departure from Hardy Weinberg Equilibrium and genotyping error. *Front. Genet.* **8**, 1–6.

**Cheng, C. S., El-Abd, Y., Bui, K., Hyun, Y.-E., Hughes, R. H., Kraus, W. E. and Truskey, G. A.** (2013). Conditions that promote primary human skeletal myoblast culture and muscle differentiation in vitro. *Am. J. Physiol. Physiol.* **306**, C385–C395.

**Cohn, R. D. and Campbell, K. P.** (2000). Molecular basis of muscular dystrophies. *Muscle and Nerve* **23**, 1456–1471.

**Cohn, R. D., van Erp, C., Habashi, J. P., Soleimani, A. A., Klein, E. C., Lisi, M. T., Gamradt, M., ap Rhys, C. M., Holm, T. M., Loeys, B. L., et al.** (2007). Angiotensin II type 1 receptor blockade attenuates TGF-β–induced failure of muscle regeneration in multiple myopathic states. *Nat. Med.* **13**, 204–210.

**Conte, G. and Gioja, L.** (1836). Scrofola del sistema muscolare. *Ann. Clin. dell'Ospedale degl'Incurabili*.

**Dadgar, S., Wang, Z., Johnston, H., Kesari, A., Nagaraju, K., Chen, Y.-W., Hill, D. A., Partridge, T. A., Giri, M., Freishtat, R. J., et al.** (2014). Asynchronous remodeling is a driver of failed regeneration in Duchenne muscular dystrophy. *J. Cell Biol.* **207**, 139–158.

**Darras, B. T., Urion, D. K. and Ghosh, P. S.** (2018). Dystrophinopathies Summary Genetic counseling GeneReview Scope. 1–35.

**Dellorusso, C., Crawford, R. W., Chamberlain, J. S. and Brooks, S. V.** (2001). Tibialis anterior muscles in mdx mice are highly susceptible to contraction-induced injury. *J. Muscle Res. Cell Motil.* **22**, 467–475.

**Doorenweerd, N., Mahfouz, A., van Putten, M., Kaliyaperumal, R., T' Hoen, P. A. C., Hendriksen, J. G. M., Aartsma-Rus, A. M., Verschuuren, J. J. G. M., Niks, E. H., Reinders, M. J. T., et al.** (2017). Timing and localization of human dystrophin isoform expression provide insights into the cognitive phenotype of Duchenne muscular dystrophy. *Sci. Rep.* **7**, 12575.

**Dwianingsih, E. K., Malueka, R. G., Nishida, A., Itoh, K., Lee, T., Yagi, M., Iijima, K., Takeshima, Y. and Matsuo, M.** (2014). A novel splicing silencer generated by DMD exon 45 deletion junction could explain upstream exon 44 skipping that modifies dystrophinopathy. *J. Hum. Genet.* **59**, 423–429.

**Eagle, M., Baudouin, S. V, Chandler, C., Giddings, D. R., Bullock, R. and Bushby, K.** (2002). Survival in Duchenne muscular dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation. *Neuromuscul. Disord.* **12**, 926–929.

**Emery, A. E. H.** (2002). The muscular dystrophies. *Lancet* **359**, 687–695.

**Felisari, G., Boneschi, F. M., Bardoni, A., Sironi, M., Comi, G. P., Robotti, M., Turconi, A. C., Lai, M., Corrao, G. and Bresolin, N.** (2000). Loss of Dp140 dystrophin isoform and intellectual impairment in Duchenne dystrophy. *Neurology* **55**, 559 LP – 564.

**Flanigan, K. M., Dunn, D. M., von Niederhausern, A., Soltanzadeh, P., Gappmaier, E., Howard, M. T., Sampson, J. B., Mendell, J. R., Wall, C., King, W. M., et al.** (2009). Mutational spectrum of DMD mutations in dystrophinopathy patients: application of modern diagnostic techniques to a large cohort. *Hum. Mutat.* **30**, 1657–1666.

**Flanigan, K. M., Ceco, E., Lamar, K. M., Kaminoh, Y., Dunn, D. M., Mendell, J. R., King, W. M., Pestronk, A., Florence, J. M., Mathews, K. D., et al.** (2013). LTBP4 genotype predicts age of ambulatory loss in duchenne muscular dystrophy. *Ann. Neurol.* **73**, 481–488.

**Fusto, A., Moyle, L.A., Gilbert P.M, Pegoraro, E.** (2019). Cored in the act: the use of models to understand core myopathies. Accepted by Disease Model and Mechanisms.

**Frontera, W. R. and Ochala, J.** (2015). Skeletal Muscle: A Brief Review of Structure and Function. *Behav. Genet.* **45**, 183–195.

**Gandhi, K. S., McKay, F. C., Cox, M., Riveros, C., Armstrong, N., Heard, R. N., Vucic, S., Williams, D. W., Stankovich, J., Brown, M., et al.** (2010). The multiple sclerosis whole blood mRNA transcriptome and genetic associations indicate dysregulation of specific T cell pathways in pathogenesis. *Hum. Mol. Genet.* **19**, 2134–2143.

**Giacopelli, F., Marciano, R., Pistorio, A., Catarsi, P., Canini, S., Karsenty, G. and Ravazzolo, R.** (2004). Polymorphisms in the osteopontin promoter affect its transcriptional activity. *Physiol. Genomics* **20**, 87–96.

**Giltay, R., Kostka, G. and Timpl, R.** (1997). Sequence and expression of a novel member (LTBP-4) of the family of latent transforming growth factor-β binding proteins. *FEBS Lett.* **411**, 164–168.

**Gimba, E. R. and Tilli, T. M.** (2013). Human osteopontin splicing isoforms: Known roles, potential clinical applications and activated signaling pathways. *Cancer Lett.* **331**, 11–17.

**Gloss, D., Moxley 3rd, R. T., Ashwal, S. and Oskoui, M.** (2016). Practice guideline update summary: Corticosteroid treatment of Duchenne muscular dystrophy: Report of the Guideline Development Subcommittee of the American Academy of Neurology. *Neurology* **86**, 465–472.

**Gomes, A. V, Potter, J. D. and Szczesna-cordary, D.** (2002). Review Article. *IUBMB Life* **54**, 323–333.

**Gordon, S.** (2003). Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**, 23–35.

**Griggs, R. C., Moxley III, R. T., Mendell, J. R., Fenichel, G. M., Brooke, M. H., Pestronk, A. and Miller, J. P.** (1991). Prednisone in Duchenne Dystrophy: A Randomized, Controlled Trial Defining the Time Course and Dose Response. *JAMA Neurol.* **48**, 383–388.

**Griggs, R. C., Miller, J. P., Greenberg, C. R., Fehlings, D. L., Pestronk, A., Mendell, J. R., Moxley 3rd,**

**R. T., King, W., Kissel, J. T., Cwik, V., et al.** (2016). Efficacy and safety of deflazacort vs prednisone and placebo for Duchenne muscular dystrophy. *Neurology* **87**, 2123–2131.

**Han, R., Rader, E. P., Levy, J. R., Bansal, D. and Campbell, K. P.** (2011). Dystrophin deficiency exacerbates skeletal muscle pathology in dysferlin-null mice. *Skelet. Muscle* **1**, 1–11.

**Haslett, J. N., Sanoudou, D., Kho, A. T., Bennett, R. R., Greenberg, S. A., Kohane, I. S., Beggs, A. H. and Kunkel, L. M.** (2002). Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 15000–15005.

**Heydemann, A., Palmer, A. A., Elizabeth, M., Heydemann, A., Ceco, E., Lim, J. E., Hadhazy, M., Ryder, P., Moran, J. L., Beier, D. R., et al.** (2010). Latent TGF- b – binding protein 4 modifies muscular dystrophy in mice Find the latest version : Latent TGF- β – binding protein 4 modifies muscular dystrophy in mice.

**Hinz, B.** (2015). The extracellular matrix and transforming growth factor-β1: Tale of a strained relationship. *Matrix Biol.* **47**, 54–65.

**Hirata, A., Masuda, S., Tamura, T., Kai, K., Ojima, K., Fukase, A., Motoyoshi, K., Kamakura, K., Miyagoe-Suzuki, Y. and Takeda, S.** (2003). Expression Profiling of Cytokines and Related Genes in Regenerating Skeletal Muscle after Cardiotoxin Injection: A Role for Osteopontin. *Am. J. Pathol.* **163**, 203–215.

**Hoffman, E. P., Brown Jr., R. H. and Kunkel, L. M.** (1987). Dystrophin: The protein product of the duchenne muscular dystrophy locus. *Cell* **51**, 919–928.

**Hoffman, E. P., Gordish-Dressman, H., McLane, V. D., Devaney, J. M., Thompson, P. D., Visich, P., Gordon, P. M., Pescatello, L. S., Zoeller, R. F., Moyna, N. M., et al.** (2013). Alterations in osteopontin modify muscle size in females in both humans and mice. *Med. Sci. Sports Exerc.* **45**, 1060–1068.

**Hogarth, M. W., Houweling, P. J., Thomas, K. C., Gordish-Dressman, H., Bello, L., Pegoraro, E., Hoffman, E. P., Head, S. I. and North, K. N.** (2017). Evidence for ACTN3 as a genetic modifier of Duchenne muscular dystrophy. *Nat. Commun.* **8**, 1–13.

**Hufton, M. and Roper, H.** (2017). Variations in Duchenne muscular dystrophy course in a multi-ethnic UK population: potential influence of socio-economic factors. *Dev. Med. Child Neurol.* **59**, 837–842.

**Humbertclaude, V., Hamroun, D., Bezzou, K., Bérard, C., Boespflug-Tanguy, O., Bommelaer, C., Campana-Salort, E., Cances, C., Chabrol, B., Commare, M.-C., et al.** (2012). Motor and respiratory heterogeneity in Duchenne patients: Implication for clinical trials. *Eur. J. Paediatr. Neurol.* **16**, 149–160.

**Jacobson, E. M., Concepcion, E., Oashi, T. and Tomer, Y.** (2005). A Graves' Disease-Associated Kozak

Sequence Single-Nucleotide Polymorphism Enhances the Efficiency of CD40 Gene Translation: A Case for Translational Pathophysiology. *Endocrinology* **146**, 2684–2691.

**Jefferies, J. L., Eidem, B. W., Belmont, J. W., Craigen, W. J., Ware, S. M., Fernbach, S. D., Neish, S. R., Smith, E. O. B. and Towbin, J. A.** (2005). Genetic predictors and remodeling of dilated cardiomyopathy in muscular dystrophy. *Circulation* **112**, 2799–2804.

**Jonathan, P., Kazunori, N. and Suzuki Katsuhiko** (2005). Characterization of inflammatory responses to eccentric exercise in humans. *Exerc Immunol Rev* 64–85.

**Juan-Mateu, J., Gonzalez-Quereda, L., Rodriguez, M. J., Baena, M., Verdura, E., Nascimento, A., Ortez, C., Baiget, M. and Gallano, P.** (2015). DMD Mutations in 576 Dystrophinopathy Families: A Step Forward in Genotype-Phenotype Correlations. *PLoS One* **10**, e0135189.

**Jungbluth, H., Sewry, C. A. and Muntoni, F.** (2011). Core myopathies. *Semin. Pediatr. Neurol.*

**Khan, N., Eliopoulos, H., Han, L., Kinane, T. B., Lowes, L. P., Mendell, J. R., Gordish-Dressman, H., Henricson, E. K., McDonald, C. M. and Investigators, E. I. and the C. D.** (2019). Eteplirsen Treatment Attenuates Respiratory Decline in Ambulatory and Non-Ambulatory Patients with Duchenne Muscular Dystrophy. *J. Neuromuscul. Dis.* **6**, 213–225.

**Klitzner, T. S., Beekman, R. H., Galioto, F. M., Jones, T. K., Manning, P. B., Morrow, W. R., Newburger, J., Moore, J. W. M., Cripe, L. H. and Colegrove, L.** (2005). Cardiovascular health supervision for individuals affected by duchenne or becker muscular dystrophy. *Pediatrics* **116**, 1569–1573.

**Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C. and Kunkel, L. M.** (1987). Complete cloning of the duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* **50**, 509–517.

**Koenig, M., Beggs, A. H., Moyer, M., Scherpf, S., Heindrich, K., Bettecken, T., Meng, G., Müller, C. R., Lindlöf, M., Kaariainen, H., et al.** (1989). The molecular basis for duchenne versus becker muscular dystrophy: Correlation of severity with type of deletion. **45**, 498–506.

**Kuraoka, M., Kimura, E., Nagata, T., Okada, T., Aoki, Y., Tachimori, H., Yonemoto, N., Imamura, M. and Takeda, S.** (2016). Serum Osteopontin as a Novel Biomarker for Muscle Regeneration in Duchenne Muscular Dystrophy. *Am. J. Pathol.* **186**, 1302–1312.

**Lamar, K.-M., Bogdanovich, S., Gardner, B. B., Gao, Q. Q., Miller, T., Earley, J. U., Hadhazy, M., Vo, A. H., Wren, L., Molkentin, J. D., et al.** (2016). Overexpression of Latent TGFβ Binding Protein 4 in Muscle Ameliorates Muscular Dystrophy through Myostatin and TGFβ. *PLOS Genet.* **12**, e1006019.

**Lamb, M. M., West, N. A., Ouyang, L., Yang, M., Weitzenkamp, D., James, K., Ciafaloni, E., Pandya, S., DiGuiseppi, C., Cunniff, C., et al.** (2016). Corticosteroid Treatment and Growth Patterns in Ambulatory Males with Duchenne Muscular Dystrophy. *J. Pediatr.* **173**, 207-213.e3.

**Li, Y., Foster, W., Deasy, B. M., Chan, Y., Prisk, V., Tang, Y., Cummins, J. and Huard, J.** (2004). Transforming Growth Factor-β1 Induces the Differentiation of Myogenic Cells into Fibrotic Cells in Injured Skeletal Muscle: A Key Event in Muscle Fibrogenesis. *Am. J. Pathol.* **164**, 1007–1019.

**LoMauro, A., Romei, M., Gandossini, S., Pascuzzo, R., Vantini, S., D'Angelo, M. G. and Aliverti, A.** (2018). Evolution of respiratory function in Duchenne muscular dystrophy from childhood to adulthood. *Eur. Respir. J.* **51**, 1701418.

**MacArthur, D. G., Seto, J. T., Chan, S., Quinlan, K. G. R., Raftery, J. M., Turner, N., Nicholson, M. D., Kee, A. J., Hardeman, E. C., Gunning, P. W., et al.** (2008). An Actn3 knockout mouse provides mechanistic insights into the association between α-actinin-3 deficiency and human athletic performance. *Hum. Mol. Genet.* **17**, 1076–1086.

**Magri, F., Govoni, A., D'Angelo, M. G., Del Bo, R., Ghezzi, S., Sandra, G., Turconi, A. C., Sciacco, M., Ciscato, P., Bordoni, A., et al.** (2011). Genotype and phenotype characterization in a large dystrophinopathic cohort with extended follow-up. *J. Neurol.* **258**, 1610–1623.

**Mamchaoui, K., Trollet, C., Bigot, A., Negroni, E., Chaouch, S., Wolff, A., Kandalla, P. K., Marie, S., Di Santo, J., St Guily, J. L., et al.** (2011). Immortalized pathological human myoblasts: towards a universal tool for the study of neuromuscular disorders. *Skelet. Muscle*.

**Many, G. M., Yokosaki, Y., Uaesoontrachoon, K., Nghiem, P. P., Bello, L., Dadgar, S., Yin, Y., Damsker, J. M., Cohen, H. B., Kornegay, J. N., et al.** (2016). OPN-a induces muscle inflammation by increasing recruitment and activation of pro-inflammatory macrophages. *Exp. Physiol.* **101**, 1285–1300.

**Mark, M. P., Prince, C. W., Oosawa, T., Gay, S., Bronckers, A. L. and Butler, W. T.** (1987). Immunohistochemical Phosphoprotein Demonstration of a 44-lcD in Developing Rat Bones1 that picture. *J. Histochem. Cytochem*.

**Markham, L. W., Kinnett, K., Wong, B. L., Woodrow Benson, D. and Cripe, L. H.** (2008). Corticosteroid treatment retards development of ventricular dysfunction in Duchenne muscular dystrophy. *Neuromuscul. Disord.* **18**, 365–370.

**Massagué, J.** (2012). TGFβ signalling in context. *Nat. Rev. Mol. Cell Biol.* **13**, 616.

**Matthews, E., Brassington, R., Kuntzer, T., Jichi, F. and Manzur, A. Y.** (2016). Corticosteroids for the treatment of Duchenne muscular dystrophy. *Cochrane Database Syst. Rev.*

**Mayer, O. H., Finkel, R. S., Rummey, C., Benton, M. J., Glanzman, A. M., Flickinger, J., Lindström, B.-M. and Meier, T.** (2015). Characterization of pulmonary function in Duchenne Muscular Dystrophy. *Pediatr. Pulmonol.* **50**, 487–494.

**McDonald, C. M., Henricson, E. K., Abresch, R. T., Han, J. J., Escolar, D. M., Florence, J. M., Duong, T., Arrieta, A., Clemens, P. R., Hoffman, E. P., et al.** (2013). The cooperative international

neuromuscular research group duchenne natural history study—a longitudinal investigation in the era of glucocorticoid therapy: Design of protocol and the methods used. *Muscle Nerve* **48**, 32–54.

**McDonald, C. M., Henricson, E. K., Abresch, R. T., Duong, T., Joyce, N. C., Hu, F., Clemens, P. R., Hoffman, E. P., Cnaan, A., Gordish-Dressman, H., et al.** (2018a). Long-term effects of glucocorticoids on function, quality of life, and survival in patients with Duchenne muscular dystrophy: a prospective cohort study. *Lancet* **391**, 451–461.

**McDonald, C. M., Gordish-Dressman, H., Henricson, E. K., Duong, T., Joyce, N. C., Jhawar, S., Leinonen, M., Hsu, F., Connolly, A. M., Cnaan, A., et al.** (2018b). Longitudinal pulmonary function testing outcome measures in Duchenne muscular dystrophy: Long-term natural history with and without glucocorticoids. *Neuromuscul. Disord.* **28**, 897–909.

**Mendell, J. R., Moxley, R. T., Griggs, R. C., Brooke, M. H., Fenichel, G. M., Miller, J. P., King, W., Signore, L., Pandya, S., Florence, J., et al.** (1989). Randomized, Double-Blind Six-Month Trial of Prednisone in Duchenne's Muscular Dystrophy. *N. Engl. J. Med.* **320**, 1592–1597.

**Mercuri, E., McDonald, C., Mayhew, A., Florence, J., Mazzone, E., Bianco, F., Decostre, V., Servais, L., Ricotti, V., Goemans, N., et al.** (2012). International workshop on assessment of upper limb function in Duchenne Muscular Dystrophy. Rome, 15-16 February 2012. *Neuromuscul. Disord.* **22**, 1025–1028.

**Meryon E** (1852). On granular and fatty degeneration of te voluntary muscle. *Medico-Chirurgical Trans.*

**Mills, M., Yang, N., Weinberger, R., Vander Woude, D. L., Beggs, A. H., Easteal, S. and North, K.** (2001). Differential expression of the actin-binding proteins, α-actinin-2 and -3, in different species: implications for the evolution of functional redundancy. *Hum. Mol. Genet.* **10**, 1335–1346.

**Muntoni, F., Torelli, S. and Ferlini, A.** (2003). Dystrophin and mutations: one gene, several proteins, multiple phenotypes. **44**, 731–740.

**Murphy-Ullrich, J. E. and Poczatek, M.** (2000). Activation of latent TGF-β by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev.* **11**, 59–69.

**Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., Gordon, S., Hamilton, J. A., Ivashkiv, L. B., Lawrence, T., et al.** (2014). Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* **41**, 14–20.

**Nelson, S. F. and Griggs, R. C.** (2011). Predicting the severity of Duchenne muscular dystrophy. *Neurology* **76**, 208 LP – 209.

**Nigro, G., Comi, L. I., Politano, L. and Bain, R. J. I.** (1990). The incidence and evolution of

cardiomyopathy in Duchenne muscular dystrophy. *Int. J. Cardiol.* **26**, 271–277.

**North, K. N. and Beggs, A. H.** (1996). Deficiency of a skeletal muscle isoform of α-actinin (α-actinin-3) in merosin-positive congenital muscular dystrophy. *Neuromuscul. Disord.* **6**, 229–235.

**North, K. N., Yang, N., Duangrurdee, W., Mills, M., Easteal, S. and Beggs, A. H.** (1999). A common nonsense mutation results in α-actinin-3 deficiency in the general population. *Nat. Genet.* **21**, 353–354.

**Onouchi, Y., Ozaki, K., Burns, J. C., Shimizu, C., Terai, M., Hamada, H., Honda, T., Suzuki, H., Suenaga, T., Takeuchi, T., et al.** (2012). A genome-wide association study identifies three new risk loci for Kawasaki disease. *Nat. Genet.* **44**, 517–521.

**Pagel, C. N., Wasgewatte Wijesinghe, D. K., Taghavi Esfandouni, N. and Mackie, E. J.** (2014). Osteopontin, inflammation and myogenesis: influencing regeneration, fibrosis and size of skeletal muscle. *J. Cell Commun. Signal.* **8**, 95–103.

**Paliwal, P., Pishesha, N., Wijaya, D. and Conboy, I. M.** (2012). Age dependent increase in the levels of osteopontin inhibits skeletal muscle regeneration. *Aging (Albany. NY).* **4**, 553–566.

**Pampaloni, F., Reynaud, E. G. and Stelzer, E. H. K.** (2007). The third dimension bridges the gap between cell culture and live tissue : Abstract : Nature Reviews Molecular Cell Biology. *Nature* **8**, 839–845.

**Pane, M., Mazzone, E. S., Fanelli, L., De Sanctis, R., Bianco, F., Sivo, S., D'Amico, A., Messina, S., Battini, R., Scutifero, M., et al.** (2014a). Reliability of the Performance of Upper Limb assessment in Duchenne muscular dystrophy. *Neuromuscul. Disord.* **24**, 201–206.

**Pane, M., Mazzone, E. S., Sormani, M. P., Messina, S., Vita, G. L., Fanelli, L., Berardinelli, A., Torrente, Y., D'Amico, A., Lanzillotta, V., et al.** (2014b). 6 Minute Walk Test in Duchenne MD Patients with Different Mutations: 12 Month Changes. *PLoS One* **9**, e83400.

**Partridge, T. A., Morgan, J. E., Coulton, G. R., Hoffman, E. P. and Kunkel, L. M.** (1989). Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature* 176–179.

**Pegoraro, E., Hoffman, E. P., Piva, L., Gavassini, B. F., Cagnin, S., Ermani, M., Bello, L., Soraru, G., Pacchioni, B., Bonifati, M. D., et al.** (2011). SPP1 genotype is a determinant of disease severity in Duchenne muscular dystrophy. *Neurology* **76**, 219 LP – 226.

**Pessina, P., Kharraz, Y., Jardí, M., Fukada, S., Serrano, A. L., Perdiguero, E. and Muñoz-Cánoves, P.** (2015). Fibrogenic Cell Plasticity Blunts Tissue Regeneration and Aggravates Muscular Dystrophy. *Stem Cell Reports* **4**, 1046–1060.

**Petrof, B. J., Stedman, H. H., Shrager, J. B., Eby, J., Sweeney, H. L. and Kelly, A. M.** (1993). Adaptations in myosin heavy chain expression and contractile function in dystrophic mouse diaphragm. *Am.*

*J. Physiol. Physiol.* C834–C841.

**Petrof, B. J., Shrager, J. B., Stedman, H. H., Kelly, A. M. and Sweeney, H. L.** (2006). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci.* **90**, 3710–3714.

**Piva, L., Gavassini, B. F., Bello, L., Fanin, M., Soraru, G., Barp, A., Ermani, M., Angelini, C., Hoffman, E. P. and Pegoraro, E.** (2012). TGFBR2 but not SPP1 genotype modulates osteopontin expression in Duchenne muscular dystrophy muscle. *J. Pathol.* **228**, 251–259.

**Porter, J. D., Khanna, S., Kaminski, H. J., Rao, J. S., Merriam, A. P., Richmonds, C. R., Leahy, P., Li, J., Guo, W. and Andrade, F. H.** (2002). A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum. Mol. Genet.* **11**, 263–272.

**Quanjer, P., Tammeling, G., Cotes, J., Pedersen, O., Peslin, R. and Yernault, J.** (1993). Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur. Respir. J.* 5–40.

**Quattrocelli, M., Spencer, M. J. and McNally, E. M.** (2017). Outside in: The matrix as a modifier of muscular dystrophy. *Biochim. Biophys. acta. Mol. cell Res.* **1864**, 572–579.

**Quinlan, K. G. R., Seto, J. T., Turner, N., Vandebrouck, A., Floetenmeyer, M., Macarthur, D. G., Raftery, J. M., Lek, M., Yang, N., Parton, R. G., et al.** (2010). α-Actinin-3 deficiency results in reduced glycogen phosphorylase activity and altered calcium handling in skeletal muscle. *Hum. Mol. Genet.* **19**, 1335–1346.

**Rando, T. A.** (2001). The dystrophin–glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. *Muscle Nerve* **24**, 1575–1594.

**Rebbeck, R. T., Karunasekara, Y., Board, P. G., Beard, N. A., Casarotto, M. G. and Dulhunty, A. F.** (2014). Skeletal muscle excitation-contraction coupling: Who are the dancing partners? *Int. J. Biochem. Cell Biol.* **48**, 28–38.

**Ricotti, V., Selby, V., Ridout, D., Domingos, J., Decostre, V., Mayhew, A., Eagle, M., Butler, J., Guglieri, M., Van der Holst, M., et al.** (2019). Respiratory and upper limb function as outcome measures in ambulant and non-ambulant subjects with Duchenne muscular dystrophy: A prospective multicentre study. *Neuromuscul. Disord.* **29**, 261–268.

**Rosenberg, A. s, Puig, M., Nagaraju, K., Hoffman, E. P., Villalta, A. S., Rao, A. V, Wakefield, L. M. and Woodcock, J.** (2015). Immune-mediated pathology in Duchenne muscular dystrophy. *Sci. Transl. Med.* **7**, 299rv4.

**Rouger, K., Le Cunff, M., Steenman, M., Potier, M.-C., Gibelin, N., Dechesne, C. A. and Leger, J. J.** (2002). Global/temporal gene expression in diaphragm and hindlimb muscles of dystrophin-

deficient (mdx) mice. *Am. J. Physiol. Physiol.* **283**, C773–C784.

**Ryder, S., Leadley, R. M., Armstrong, N., Westwood, M., de Kock, S., Butt, T., Jain, M. and Kleijnen, J.** (2017). The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. *Orphanet J. Rare Dis.* **12**, 79.

**Ryu, J. K., Davalos, D. and Akassoglou, K.** (2009). Fibrinogen signal transduction in the nervous system. *J. Thromb. Haemost.* **7**, 151–154.

**Sadoulet-Puccio, H. M. and Kunkel, L. M.** (1996). Dystrophin and Its Isoforms. *Brain Pathol.* **6**, 25–35.

**Saitoh, Y., Kuratsu, J., Takeshima, H., Yamamoto, S. and Ushio, Y.** (1995). Expression of osteopontin in human glioma. Its correlation with the malignancy. *Lab. Investig.* 55–63.

**Schiller, N. B., Shah, P. M., Crawford, M., DeMaria, A., Devereux, R., Feigenbaum, H., Gutgesell, H., Reichek, N., Sahn, D., Schnittger, I., et al.** (1989). Recommendations for Quantitation of the Left Ventricle by Two-Dimensional Echocardiography. *J. Am. Soc. Echocardiogr.* **2**, 358–367.

**Silversides, C. K., Webb, G. D., Harris, V. A. and Biggar, D. W.** (2003). Effects of deflazacort on left ventricular function in patients with Duchenne muscular dystrophy. *Am. J. Cardiol.* **91**, 769–772.

**Smith, A. S. T., Davis, J., Lee, G., Mack, D. L., Kim, D.-H. and Edu, D.** (2016). Muscular dystrophy in a dish: engineered human skeletal muscle mimetics for disease modeling and drug discovery. *Drug Discov Today* **21**, 1387–1398.

**Smith, A. S. T., Davis, J., Lee, G. and Mack, D. L.** (2017). Muscle Mimetics for Disease Modeling and Drug Discovery. **21**, 1387–1398.

**Spurney, C., Shimizu, R., Morgenroth, L. P., Kolski, H., Gordish-Dressman, H., Clemens, P. R. and Investigators, the C.** (2014). Cooperative international neuromuscular research group duchenne natural history study demonstrates insufficient diagnosis and treatment of cardiomyopathy in duchenne muscular dystrophy. *Muscle Nerve* **50**, 250–256.

**Sterner-Kock, A., Thorey, I. S., Koli, K., Wempe, F., Otte, J., Bangsow, T., Kuhlmeier, K., Kirchner, T., Jin, S., Keski-Oja, J., et al.** (2002). Disruption of the gene encoding the latent transforming growth factor-β binding protein 4 (LTBP-4) causes abnormal lung development, cardiomyopathy, and colorectal cancer. *Genes Dev.* **16**, 2264–2273.

**Suelves, M., Vidal, B., Serrano, A. L., Tjwa, M., Roma, J., López-Alemany, R., Luttun, A., de Lagrán, M. M., Díaz, M. À., Jardí, M., et al.** (2007). uPA deficiency exacerbates muscular dystrophy in <em>MDX</em> mice. *J. Cell Biol.* **178**, 1039 LP – 1051.

**Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O. and Allen, R. .** (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev. Biol. J.* 114–128.

**Tidball, J. G., Dorshkind, K. and Wehling-Henricks, M.** (2014). Shared signaling systems in myeloid

cell-mediated muscle regeneration. *Development* **141**, 1184 LP – 1196.

**Uaesoontrachoon, K., Yoo, H.-J., Tudor, E. M., Pike, R. N., Mackie, E. J. and Pagel, C. N.** (2008). Osteopontin and skeletal muscle myoblasts: Association with muscle regeneration and regulation of myoblast function in vitro. *Int. J. Biochem. Cell Biol.* **40**, 2303–2314.

**Uaesoontrachoon, K., Wasgewatte Wijesinghe, D. K., Mackie, E. J. and Pagel, C. N.** (2013). Osteopontin deficiency delays inflammatory infiltration and the onset of muscle regeneration in a mouse model of muscle injury. *Dis. Model. Mech.* **6**, 197–205.

**van den Bergen, J. C., Ginjaar, H. B., Niks, E. H., Aartsma-Rus, A. and Verschuuren, J. J. G. M.** (2014). Prolonged ambulation in duchenne patients with a mutation amenable to exon 44 skipping. *J. Neuromuscul. Dis.* **1**, 91–94.

**van den Bergen, J. C., Hiller, M., Böhringer, S., Vijfhuizen, L., Ginjaar, H. B., Chaouch, A., Bushby, K., Straub, V., Scoto, M., Cirak, S., et al.** (2015). Validation of genetic modifiers for Duchenne muscular dystrophy: a multicentre study assessing <em>SPP1</em> and <em>LTBP4</em> variants. *J. Neurol. Neurosurg. & Psychiatry* **86**, 1060 LP – 1065.

**Van Dorn, C. S., Puchalski, M. D., Weng, H. Y., Bleyl, S. B., Butterfield, R. J. and Williams, R. V.** (2018). DMD mutation and LTBP4 haplotype do not predict onset of left ventricular dysfunction in Duchenne muscular dystrophy. *Cardiol. Young* **28**, 910–915.

**Vetrone, S. A., Montecino-Rodriguez, E., Kudryashova, E., Kramerova, I., Hoffman, E. P., Liu, S. D., Miceli, M. C. and Spencer, M. J.** (2009). Osteopontin promotes fibrosis in dystrophic mouse muscle by modulating immune cell subsets and intramuscular TGF-β. *J. Clin. Invest.* **119**, 1583–1594.

**Vianello, S., Pantic, B., Fusto, A., Bello, L., Galletta, E., Borgia, D., Gavassini, B. F., Semplicini, C., Sorarù, G., Vitiello, L., et al.** (2017). SPP1 genotype and glucocorticoid treatment modify osteopontin expression in Duchenne muscular dystrophy cells. *Hum. Mol. Genet.* **26**,.

**Victor Dubowitz and Sewry, C. A.** (2007). *Muscle Biopsy: A Pratical Approach. 3rd Edition*. 3rd ed. Elsevier Limited.

**Wang, R. T., Barthelemy, F., Martin, A. S., Douine, E. D., Eskin, A., Lucas, A., Lavigne, J., Peay, H., Khanlou, N., Sweeney, L., et al.** (2018). DMD genotype correlations from the Duchenne Registry: Endogenous exon skipping is a factor in prolonged ambulation for individuals with a defined mutation subtype. *Hum. Mutat.* **39**, 1193–1202.

**Weiss, R. B., Vieland, V. J., Dunn, D. M., Kaminoh, Y. and Flanigan, K. M.** (2018). Long-range genomic regulators of THBS1 and LTBP4 modify disease severity in duchenne muscular dystrophy. *Ann. Neurol.* **84**, 234–245.

**Wong, B. L. Y. and Christopher, C.** (2002). Corticosteroids in Duchenne Muscular Dystrophy: A Reappraisal. *J. Child Neurol.* **17**, 183–190.

**Wynn, T. A.** (2015). Type 2 cytokines: mechanisms and therapeutic strategies. *Nat. Rev. Immunol.* **15**, 271.

**Yaffe, D., Makover, A., Lederfein, D., Rapaport, D., Bar, S., Barnea, E. and Nudel, U.** (1992). Multiple prooducts of the Duchenne muscular dystrophy gene. *Symp Soc Exp Biol* **46**, 179–188.

**Yan, Z., Bai, X. C., Yan, C., Wu, J., Li, Z., Xie, T., Peng, W., Yin, C. C., Li, X., Scheres, S. H. W., et al.** (2015). Structure of the rabbit ryanodine receptor RyR1 at near-atomic resolution. *Nature*.

**Yang, N., MacArthur, D. G., Gulbin, J. P., Hahn, A. G., Beggs, A. H., Easteal, S. and North, K.** (2003). ACTN3 Genotype Is Associated with Human Elite Athletic Performance. *Am. J. Hum. Genet.* **73**, 627–631.

**Yin, H., Price, F. and Rudnicki, M. A.** (2013). Satellite cells and the muscle stem cell niche. *Physiol. Rev.* **93**, 23–67.

**Yokosaki, Y., Nariaki, M., Tomohiro, S., Isao, M., Holm, S., Shigeki, H., Yoshiki, S., Michio, Y., Yasuyuki, T. and Sheppard, D.** (2002). The Integrin α 9 β 1 Binds to a Novel Recognition Sequence (SVVYGLR) in the Thrombin-cleaved Amino-terminal Fragment of Osteopontin. *J. Biol. Chem.* **274**, 36328–36334.

**Zanotti, S., Gibertini, S., Di Blasi, C., Cappelletti, C., Bernasconi, P., Mantegazza, R., Morandi, L. and Mora, M.** (2011). Osteopontin is highly expressed in severely dystrophic muscle and seems to play a role in muscle regeneration and fibrosis. *Histopathology* **59**, 1215–1228.

**Zhou, L., Porter, J. D., Cheng, G., Gong, B., Hatala, D. A., Merriam, A. P., Zhou, X., Rafael, J. A. and Kaminski, H. J.** (2006). Temporal and spatial mRNA expression patterns of TGF-β1, 2, 3 and TβRI, II, III in skeletal muscles of mdx mice. *Neuromuscul. Disord.* **16**, 32–38.

# Appendix A

| Performance of the Upper Limb Module per DMD 1.2 (PUL per DMD) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Nome o ID:** **Data di nascita:**<br><br>**Data della valutazione: Chirurgia rachide S N (cerchiare) Data:**<br><br>**Lato dominante: Valutatore/ firma:**<br><br>**Retrazioni gomito: Destra: Sinistra:**<br><br>**Deambulante / non-deambulante (cerchiare) Steroidi / no steroidi (cerchiare)** | | | | | | | | |
| **Item d'ingresso – iniziare con item A per identificare il punto di partenza per i test successivi. Cerchiare il punteggio per ciascun item.** | | | | | | | | |
| **Item** | **Descrizione** | **0** | **1** | **2** | **3** | **4** | **5** | **6** |
| **A.** | **Item d'ingresso** | Nessuna funzionalità utile delle mani | Capace di usare le mani per tenere una penna, tirar su una moneta o guidare una carrozzina elettronica | Capace di sollevare 1 o 2 mani alla bocca ma non un bicchiere con un peso di 200gr alla bocca | Capace di sollevare un bicchiere con un peso di 200gr alla bocca usando 2 mani se necessario | Capace di sollevare entrambe le braccia all'altezza delle spalle con o senza movimenti di compenso. Gomito piegato o in estensione | Capace di sollevare entrambe le braccia insieme sopra la testa solo con la flessione dei gomiti (accorciando o la circonferenza /usando muscoli accessori) | Capace di abdurre entrambe le braccia simultanea mente con gomiti estesi in cerchio fino a toccarsi sopra la testa. |
| **Se capace di score 4 o 5 o 6 su item A , iniziare con item B.** | | | | | | | | |
| **Livello Alto-Dimensione della spalla** | | | | | | | | |
| **Item** | **Descrizione** | **0** | **1** | **2** | **3** | **4** | **5** | **6** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **B.**<br><br>**Sinistra / Destra** | **Abduzione della spalla a livello della spalla**<br>Gomito a livello spalla | Non riesce | Riesce senza pesi | 200gr X | 500gr | 1000gr | **Segnare /cerchiare la casella solo se completa B, C senza compensi.** | |
| **C.**<br>**Sinistra / Destra** | **Abduzione della spalla sopra livello della spalla**<br>Gomito a livello occhi | Non riesce | Riesce senza pesi X | 200gr | 500gr | 1000gr | | |
| **D.**<br><br>**Sinistra / Destra** | **Flessione della spalla a livello della spalla**<br>Gomito a livello spalla | Non riesce | Riesce senza pesi | 200gr | 500gr X | 1000gr | | |
| **E.**<br><br>**Sinistra / Destra** | **Flessione della spalla sopra livello della spalla**<br>Gomito a livello | Non riesce | Riesce senza pesi | 200gr X | 500gr | 1000gr | | |

| **Performance of the Upper Limb Module for DMD 1.2 (PUL per DMD)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Livello Medio-Dimensione del gomito** | | | | | | | | |
| **Eseguire questi test su tutti I pazienti** | | | | | | | | |
| **Item** | **Descrizione** | **0** | **1** | **2** | **3** | **4** | **5** | **6** |
| **F. Sinistra/ Destra Entrambe** | Mano/i alla bocca | Non riesce | Riesce ai portare alla bocca 50gr nel bicchiere usando 2 mani | Riesce a portare alla bocca 200gr o con 2 mani o usando una mano X | Riesce a portare alla bocca 200gr con 1 mano e senza | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | e sostegno del gomito | sostegno del gomito | | | |
| **G.** | Mano/i dalle ginocchia al tavolo | Non riesce | Porta solo le dita di entrambe le mani o una mano sola al tavolo | Porta entrambe le mani sul tavolo ma non insieme | Porta entrambe le mani sul tavolo insieme | | | |
| **H. Sinistra/ Destra** | Spostare peso sul tavolo | Non riesce | Sposta 100g dal cerchio esterno a quello centrale | Solleva 100gr dal cerchio esterno a quello centrale senza compensi | Solleva 200gr dal cerchio esterno a quello centrale senza compensi | Solleva 500gr dal cerchio esterno a quello centrale senza compensi | Solleva un 1kg dal cerchio esterno a quello centrale, avambraccio fuori dal tavolo | |
| **I. Sinistra/ Destra Entrambe** | **Sollevare barattoli leggeri** | Solleva 0 | Solleva 1° (esterno) | Solleva 2° | Solleva 3° (centrale) | Solleva 4° | Solleva 5° (più lontano dal lato scelto) | **Tempo per sollevare 5 barattoli =** 6 0 1– – – **sec/min** |
| **J. Sinistra/ Destra Entrambe** | Sollevare barattoli pesanti | Solleva 0 | Solleva 1° (esterno) | Solleva 2° | Solleva 3° (centrale) | Solleva 4° | Solleva 5° (più lontano dal lato scelto) | **Tempo per sollevare 5 barattoli =** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 4 78_. _ _ **sec/min** |
| **K. Sinistra/ Destra Entrambe** | **Impilare barattoli leggeri** | Non riesce a impilare il 2° | Riesce di impilare il 2° barattolo | Riesce a impilare il 3° barattolo | Riesce a impilare il 4° barattolo | Riesce di impilare il 5° barattolo | | **Tempo per impilare 5 i barattoli** 7_. _ _ 97 **sec/min** |
| **L. Sinistra/ Destra Entrambe** | **Impilare barattoli pesanti** | Non riesce a impilare il 2° | Riesce a impilare il 2° barattolo | Riesce a impilare il 3° barattolo | Riesce a impilare il 4° barattolo | Riesce a impilare il 5° barattolo | | **Tempo per impilare 5 i barattoli** 6 67_. _ _ **sec/min** |
| **M.** | Togliere il coperchio ad un contenitore | Non riesce a aprire | Apre completamente | | | | | |
| **N.** | Strappare un foglio di carta | Non tiene il foglio o non lo strappa | Tiene il foglio di carta non piegato ma non lo strappa | Strappa il foglio non piegato | Strappa il foglio piegato in 2, iniziando dal lato piegato | Strappa il foglio piegato in 4, iniziando dal lato piegato | | |

| Performance of the Upper Limb Module for DMD 1.2 (PUL per DMD) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Dimensione distale del polso e della mano Eseguire questi test su tutti i pazienti** | | | | | | | |
| | | **0** | **1** | **2** | **3** | **4** | |
| **O.** **Sinistra/ Destra** | Tracciare un percorso | Con matita nella mano non riesce a tenerla o fare un segno scritto | Riesce a tenere la matita e a fare un segno scritto sulla carta | Riesce a seguire il percorso per almeno 5cm ma non a completarlo | Riesce a completare il percorso ma si deve fermare e/o solleva la matita dal foglio | Riesce a tirar su la matita ed a completare il percorso senza fermarsi o sollevare la matita | |
| **P.** | Premere la luce a pulsante | Non riesce ad accendere la lampada con le dita di entrambe le mani | Riesce ad accendere momentaneamente la lampada con dita di entrambe le mani | Riesce ad accendere momentaneamente la lampada con dita di una mano | Riesce ad accendere permanentemente la lampada con dita di una mano | | |
| **Q.** **Sinistra/ Destra** | Supinazione | Non riesce a sollevare la lampada | Solleva la lampada ma non riesce a girare la mano | Solleva la lampada e gira la mano in maniera incompleta | Solleva la lampada e gira la mano completamente con movimenti di compenso | Solleva la lampada e gira la mano completamente senza movimenti di compenso | |
| **R.** **Sinistra/ Destra** | Sollevare le monete | Non riesce a sollevare una moneta | Solleva una moneta | Riesce a sollevare e tenere 3 monete nella mano | Riesce a sollevare e tenere 6 monete nella mano | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **S.**<br><br>**Sinistra/ Destra** | Posizionare dito sui numeri del diagramma | Non riesce a sollevare o strisciare il dito sul diagramma | Non solleva dito per posizionarlo sul disegno ma riesce a strisciarlo tra almeno 2 caselle | Solleva il dito e lo posiziona in maniera imprecisa su tutti i numeri | Solleva il dito e lo posiziona in sequenza sul diagramma senza toccare le linee | |
| **T.**<br>**Sinistra/ Destra** | Pinza a 2 punti | Non usa una pinza a due dita<br>5 gr 10 gr | Usa una pinza a due dita ma non solleva peso<br>5 gr 10 gr | Usa una pinza a due dita e solleva peso<br>5 gr 10 gr | Cerchiare il peso 5 gr e/o 10 gr usato. Per score 1 si intende la capacità di posizionare pollice e dito con posizione a pinza. | |
| **U.**<br>**Sinistra/ Destra** | Pinza a tre punti | Non riesce ad usare una pinza a 3 punti<br><br>5 gr 10 gr | Riesce ad usare una pinza a 3 punti ma non solleva peso<br>5 gr 10 gr | Riesce ad usare una pinza a 3 punti e solleva peso<br>5 gr 10 gr | Cerchiare il peso 5 gr e/o 10 gr usato. Per score 1 si intende la capacità di posizionare pollice e due dita con posizione a pinza. | |
| **V.**<br>**Sinistra/ Destra**<br>☐ mano chiusa, dita flesse<br>☐ mano aperta, dita estese | Grip a chiave/pollice | Non riesce a usare grip a pollice o piegare la punta del pollice | Non riesce a usare grip a pollice ma piega la punta del pollice 5 gr 10gr | Riesce a usare grip a pollice ma non solleva il peso<br><br>5 gr 10gr | Riesce a usare grip a pollice e solleva il peso<br><br>5 gr 10gr | Assegnare punteggio per 5 gr e/o 10 gr Annotare le modifiche di grip usate |