The \textit{mdx} mutation in the 129/Sv background results in a milder phenotype: Transcriptome comparative analysis searching for the protective factors.


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\textbf{Running: the \textit{mdx} mutation in the 129/Sv background}

\textbf{Key words:} \textit{mdx}, animal models, genetic background, neuromuscular disease

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Summary  (300 words)

The mdx mouse is a good genetic and molecular murine model for Duchenne Muscular Dystrophy (DMD), a progressive and devastating muscle disease. However, this model is inappropriate for testing new therapies due to its mild phenotype. Here, we transferred the mdx mutation to the 129/Sv strain to create a more severe model for DMD. Unexpectedly, functional analysis of the first three generations of mdx\textsuperscript{129} showed a progressive amelioration of the phenotype, associated to less connective tissue replacement, and more regenerating fibers than the original mdx\textsuperscript{C57BL}. Transcriptome comparative analysis was performed to try to identify what is protecting this new model from the dystrophic characteristics. The mdx\textsuperscript{C57BL} presents three times more differentially expressed genes (DEGs) than the mdx\textsuperscript{129} (371 and 137 DEGs respectively). However, both models present more over expressed genes than under-expressed, indicating that the dystrophic and regenerative alterations are more associated with the activation rather than the repression of genes. The effects of the background change were present since the first generation in the mdx\textsuperscript{129}. As to the functional categories, the DEGs of both mdx models showed a predominance of immune system genes. Excluding this category, the mdx\textsuperscript{129} model showed a decreased participation of the endo/exocytic pathway and homeostasis categories, and an increased participation of the extracellular matrix and enzymatic activity categories. The most significant DEGs exclusively expressed in the mdx\textsuperscript{129} were the up regulated Spp1 and Il1rn genes. Spp1 is a known DMD prognostic biomarker, and our data indicate that its up regulation can benefit the phenotype. Il1rn encodes an interleukin-1 antagonist, and the inhibition of its pro-inflammatory and pro-fibrotic properties could act protecting the mdx\textsuperscript{129} animals from the inflammatory process. Modeling the expression of the DEGs involved in the mdx mutation with a benign course should be tested as a possible therapeutic target for the dystrophic process.
Introduction

Neuromuscular disorders are a heterogeneous group of genetic diseases, causing progressive loss of motor ability. More than 30 genetically defined forms are recognized and in the last decade, mutations in several genes have been reported, resulting in deficiency or loss of function of different important muscle proteins.

Duchenne muscular dystrophy (DMD) is the most common and severe human muscular dystrophy, affecting 1 in 3500 male births. It is caused by mutations in the dystrophin gene which result in the absence of this important sarcolemmal protein, and consequent muscle degeneration. The clinical course of DMD is severe and progressive, starting with muscular weakness at the age of five years, loss of ambulation around 12 years; without special care, death occurs due to respiratory failure or cardiomyopathy in the late teens (Dubowitz et al., 2013). There is no effective cure for patients suffering from this type of dystrophy.

Several animal models manifesting phenotypes observed in neuromuscular diseases have been identified in nature or generated in laboratory. These models generally present physiological alterations observed in human patients and can be used as important tools for pathophysiological studies and for therapy testing (Vainzof et al., 2008). The mdx mouse is the most widely used animal model for DMD, bearing a nonsense point mutation in exon 23 of the dystrophin gene which causes lack of this protein in the skeletal muscle (Sicinski et al., 1989). However, differently from human DMD patients, the mdx presents a mild phenotype, with normal lifespan and reproductive capacity (Bulfield et al., 1984). Therefore, this model is not effective in clinical trials to track possible functional benefits of tested therapies.

Studies in animal models, by the introduction of a human pathogenic mutation in distinct mouse backgrounds, have shown differences in phenotypical manifestation according to the mouse strain. Lima et al. (2010) introduced the mgΔ mutation from a mouse model for Marfan syndrome in two different genetic backgrounds: C57BL/6 and 129/Sv. The animals with 129/Sv background presented a more severe and earlier phenotype than those with C57BL/6 background.

Considering that the increased severity of muscle damage is extremely useful in assessing how effective a novel therapy might be at halting human disease and in order to obtain a more reliable animal model for DMD, we decided to transfer the mdx mutation to the 129/Sv background expecting that the resulting animals would present a more severe DMD phenotype.

Results

Evidences from the functional evaluations

As shown in figure 1, in the first generation of mdx129 (F1) forelimbs bar test, the animals already showed more resistance than mdxC57BL animals, yet, the results were
significant only in the ages 2, 4 and 5 months. In the grip strength test, F1 animals are stronger than \textit{mdxC57BL} mice, but the results are only significant at 30 days, three and four months. The second \textit{mdx}^{129} generation (F2) showed improved performance in all tests compared to \textit{mdxC57BL}. In both bar tests, F2 mice hang on the bar for the whole time of the test (60 seconds), and the values were significant at all ages. The strength was also increased in this group. Observing the third \textit{mdx}^{129} generation (F3) results, we can confirm the progressive increase both in resistance and strength of \textit{mdx}^{129} animals. The F3 mice hang on the bar for 60 seconds with no sign of fatigue and grip strength results are better than F1 and F2. The same results were observed in the test applied for the four limbs resistance, and hind limb strength.

Therefore, the observed results were the opposite of previously described by Lima et al. (2010) and what we were expecting. Considering the amelioration of the \textit{mdx} phenotype in the 129/Sv background, we hypothesized that factors in this strain could act protecting the \textit{mdx}^{129} from the dystrophic effect. Consequently, the study was directed to identify these factors as possible positive modifiers of the dystrophic phenotype.

\textbf{Muscle Analyses}

\textbf{Evidence from the histological analysis}

Histological analysis showed that the degenerative/regenerative processes were similar in all \textit{mdxC57BL} mice as compared to the three generations of \textit{mdx}^{129} mice. In all strains, almost 100% of centrally nucleated fibers were identified. However, we could find some regeneration foci in F1, F2 and F3 (figure 2).

\textbf{Evidence from the analysis of regenerating fibers}

In order to quantify the regenerating fibers, we evaluated the number of positive fibers for developmental myosin. The three generations of \textit{mdx}^{129} showed a significant increase in the number of these fibers, suggesting a more active regeneration (figure 2, table 1).

\textbf{Evidence of reduced degeneration}

The analysis of the connective tissue replacement, measured by the picrossirius staining, showed a statistically significant reduction in the amount of endomysial and perimysial connective tissues in the three generations of \textit{mdx}^{129}, in comparison to the \textit{mdxC57BL} of the same age (figure 2, table 1).

\textbf{Evidence of the inflammatory process}

Inflammatory infiltrate in skeletal muscle is a hallmark of neuromuscular diseases. The predominant inflammatory infiltrate is composed of macrophages and monocytes, which can be identified by the CD11b (integrin M) marker, a cell-surface protein associated with those cells.
Analysis of the inflammatory cells, as recognized by the CD11b antibody, showed a similar proportion of positive fibers in the mdx\textsuperscript{C57BL}, as well as in the three lineages of mdx\textsuperscript{129}, at six months of age, with no statistically differences among them in F1 and F2. In F3, a discrete reduction of inflammation could be detected with a significance of p=0.05.

**Evidence from transcriptome analyses**

We used expression microarray to investigate \textit{mdx}\textsuperscript{129} transcriptome comparing it to \textit{mdx}\textsuperscript{C57BL} and each of these models to their same background wild type.

**C57BL and 129/Sv background comparison**

First, we compared both wild types to verify the difference level between them. In the comparison C57BL x 129/Sv we found 44 differentially expressed genes (DEGs), and 13 DEGs among the muscle expressed genes. The majority of these DEGs were downregulated in the 129/Sv in comparison to the C57BL (table 2).

**Comparing both mdx models**

Comparing C57BL and \textit{mdx}\textsuperscript{C57BL}, we found 371 DEGs: 320 upregulated and 51 downregulated in \textit{mdx}\textsuperscript{C57BL}. When comparing 129/Sv with \textit{mdx}\textsuperscript{129} F3, we found 137 DEGs: 130 upregulated and 7 downregulated in \textit{mdx}\textsuperscript{129} F3 mice. In both backgrounds, the number of upregulated genes was higher than the downregulated genes (table 2).

The DEGs were classified according to their functional categories, and we observed that the immune system category was the most significant, gathering 60% of the genes in C57BL x \textit{mdx}\textsuperscript{C57BL} and 80% of the genes in 129/Sv x \textit{mdx}\textsuperscript{129}.

Because this category is so predominant, we decided to remove it in order to verify the significance of the participation of other categories in the two \textit{mdx} models (figure 3).

Both models showed corresponding categories, except homeostasis, which only appear in C57BL x \textit{mdx}\textsuperscript{C57BL}. Otherwise, the proportion in which these categories appear is different. Genes related to endo/exocytic pathways showed a decreased expression in 129/Sv x \textit{mdx}\textsuperscript{129}, while an increased expression in genes of other categories, such as extracellular matrix, binding to molecules/cells and enzymatic activity.

**Comparing the effect of the increasing 129/Sv background**

We also compared each generation of \textit{mdx}\textsuperscript{129} to the 129/Sv wild type. The generations \textit{mdx}\textsuperscript{129} F1, \textit{mdx}\textsuperscript{129} F2 and \textit{mdx}\textsuperscript{129} F3 were also compared between themselves. The number of genes in each comparison was small, suggesting the generations are very similar.

**Comparing both mdx models for genes expressed in the muscle**
To better visualize the changes in the $mdx^{129}$ expression profile, we filtered the lists $C57BL \times mdx^{C57BL}$, $129/Sv \times mdx^{129}$ F3 and $mdx^{C57BL} \times mdx^{129}$ F3 for genes that, according to the literature, act in the skeletal muscle. We found 85 DEGs that were exclusive to $C57BL \times mdx^{C57BL}$, 13 DEGs unique to $129/Sv \times mdx^{129}$ F3 and 12 DEGs exclusive to $mdx^{C57BL} \times mdx^{129}$ F3 (figure 4).

Of the 13 genes exclusively expressed in $129/Sv \times mdx^{129}$ (table 3), all of them presented with an up regulated expression, and two important DEGs were identified: $Spp1$ and $Ilrn$.

Comparing $mdx^{C57BL} \times mdx^{129}$ we found 12 exclusive genes (table 3), where $Klk3$ showed the more up regulated expression and $Mup1$, the more down regulated expression.

**Comparing the predominant pathways in the two $mdx$ models**

We also examined gene pathways generated with the DEGs in the lists $C57BL \times mdx^{C57BL}$ and $129/Sv \times mdx^{129}$ and each comparison generated only one significant pathway, which was related to muscle development (figure 5). The pathway generated by $129/Sv \times mdx^{129}$ was formed by only six molecules, but all of them were also present in the $C57BL \times mdx^{C57BL}$ pathway, although this one showed the involvement of more genes. Therefore, the same pathway is involved in both models, however, including a different number of molecules.

**Discussion**

Studies in animal models are essential for testing therapies, mainly in diseases still with no effective cure, as Duchenne Muscular Dystrophy. Since the most common animal model for DMD study, the $mdx$ mouse, shows a very mild phenotype, we decided to create a $mdx$ model with a different genetic background, expecting that the resulting animals would present a more severe DMD phenotype. Surprisingly, we obtained the opposite results, with the $mdx^{129}$ mice presenting a significantly amelioration of the phenotype in all the functional tests, in the successive generations, when compared to the $mdx^{C57BL}$. We could attribute this crescent improvement of the phenotype to the increase in the proportion of the 129 background. Additional observations from the histological analysis showed that $mdx^{129}$ enters the degenerative process later than $mdx^{C57BL}$ and has a longer regenerative process, compatible with a more benign phenotype. These results were confirmed with a higher number of regenerating developmental myosin positive fibers, and a lower proportion of connective tissue observed in the $mdx^{129}$ strain.

We then used expression microarrays to investigate $mdx^{129}$ transcriptome comparing it to $mdx^{C57BL}$ in adult six-months old animals, when the disease is already established.
A first interesting observation was the similarity of the C57BL versus the 129/Sv wild backgrounds, showing a small number of DEGs among the mapped genes. Besides, we did not find among the DEGs expressed in the muscle anyone who could be, somehow, associated to a directly related muscular function that could eventually provide protection against the dystrophy.

As to the comparison between the complete transcriptome of the two mdx strains, we verified that the number of DEGs is about three times higher in mdx$^{C57BL}$ than in mdx$^{129}$ when compared to wild type from the same background animals. Nevertheless, in both backgrounds the dystrophin gene mutation causes more up regulation than down regulation of gene’s expression. These results suggest that, in the new background, the dystrophin mutation clearly induced fewer mechanisms of action that could be responsible for the installation and maintenance of the dystrophic process.

The comparison of the three generations of mdx$^{129}$ showed a small number of DEGs, and we could therefore conclude that in the expression level, the background effect is present since the first generation, and it does not increase with 129/Sv background.

In both models, the participation of genes involved in the immune system was clearly predominant, which was expected, once chronic inflammation is a dystrophic muscle characteristic (Porter et al., 2002). The analysis of inflammation using a specific marker confirmed that both the mdx$^{C57BL}$ as well as the mdx$^{129}$ presented similar pattern of inflammation at the age of six months. Nevertheless, when excluding this category, some differences were noted between the two dystrophin models, including a reduction in the endo/exocytic pathway but an increase in the participation of the extracellular matrix, binding to molecules/cells and enzymatic activity pathways in the mdx$^{129}$. Vesicle trafficking is a necessary process for membrane repair (Han, 2011) and several studies have shown its importance in the dystrophic phenotype (He et al., 2012; Swaggart et al., 2014). Our findings suggest that the decreased participation of vesicle-related genes in mdx$^{129}$ animals is due to a more stable membrane, which can present, consequently, better fiber conservation and functional performance. The membrane stability can also explain why the homeostasis category disappeared, once repair mechanism is calcium dependent (Reddy et al., 2001). Among the functional categories that increased in mdx$^{129}$, the most remarkable was the extracellular matrix. As seen on histological slides, regeneration is present in these animals up to six months, consequently, matrix remodeling is probably occurring, which can explain the increased participation of genes related to this process (Gillies and Lieber, 2011).

When the analysis was more focused on genes expressed in the muscle, we identified DEGs that were exclusive for some of the compared strains. Of the 13 genes exclusively expressed in 129/Sv x mdx$^{129}$, two deserve to be highlighted: the up regulated Spp1 and Il1rn. Spp11 gene codes for osteopontin (OPN), which can be found as an extracellular matrix component and a soluble molecule with cytokine properties (O’regan and Berman, 2000) and has been described as a dystrophic and injured muscle component (Haslett et al., 2002; Porter et al., 2002; Hirata et al., 2003). Osteopontin roles in muscle
degeneration and regeneration and its effects in muscular dystrophy are not yet clear. Studies about its effects in dystrophy are divergent. In a study using knockout mice for OPN and dystrophin, it was observed that lack of OPN increased muscle strength and decreased fibrosis in the double knockout model, suggesting OPN over expression might contribute to the disease aggravation (Vetrone et al., 2009). On the other hand, the polymorphism rs28357094 (DMD prognostic biomarker) on the Spp1 promoter is associated with DMD phenotype variation. Patients carrying the rare G allele present decreased muscle strength and earlier loss of ambulation than patients carrying the T allele (Pegoraro et al., 2011). In transfected HeLa cells, this polymorphism causes a decrease in Spp1 mRNA expression, which would consequently, result in lower levels of OPN. Therefore, reduction in Spp1 expression would be associated to a worse phenotype, hence T allele carriers, would count on the protective and pro-regenerative OPN effects.

The results observed in our more benign mdx\textsuperscript{129} model, expressing more Spp1 than the mdx\textsuperscript{C57BL}, are compatible with the last observations on the role of this gene as a prognostic biomarker in humans, since it is more expressed in the mildly affected model. Additionally, this over expression could be associated to a better regeneration, since in another study performed in our lab (Almeida, C. F. and col, unpublished data), we found an over expression of Spp1 in the youngest animals of the mdx\textsuperscript{C57BL} lineage.

\textit{Il1rn} gene codes for IL-1Ra, an interleukin-1 antagonist. Considering interleukins profibrotic and pro-inflammatory effects, the increased expression of its antagonist suggests that mdx\textsuperscript{129}F3 animals could be more protected from the inflammatory process caused by these molecules.

Among the DEGs exclusive in the comparison of mdx\textsuperscript{C57BL} x mdx\textsuperscript{129} the more up regulated gene was Klk3 and the more down regulated gene was Mup1. Klk3 codes for PSA (prostate-specific antigen) which is a prostate cancer biomarker (Amaro et al., 2014), that being, we could not find a correlation between this gene and mdx\textsuperscript{129} phenotype. Mup1 is part of a gene family involved in chemical communication among animals (mice and rats) (Kumar et al., 2014) and few studies were conducted about its metabolic functions. Hui et al. (2009) observed that the skeletal muscle was a major target for Mup1. In their study with obese mice, which present lower levels of the protein, partial level corrections of the protein alleviated insulin resistance and glucose intolerance, ameliorating skeletal muscle mitochondrial function. In our study, mdx\textsuperscript{129} presented a significant decrease of Mup1 expression than mdx\textsuperscript{C57BL}, suggesting a beneficial effect of this lower expression in their phenotype. More studies will be needed to explain Klk3 and Mup1 roles in muscle function.

In conclusion, modeling the expression of these differentially expressed genes, involved in the benign course for the mdx mutation, in particular, the Spp1 gene, should be tested as possible therapeutic targets for the dystrophic process.
Materials and methods

Animals

The 129/Sv male mice were obtained from the ICB USP experimentation housing facility, while the mdx females were obtained from the Center for Human Genome and Stem Cell Researches (IB USP) experimentation housing facility. The animals were kept under controlled temperature and light conditions and were fed with pellets and water “ad libitum”. All experimental procedures were analyzed and approved by the Institute of Biosciences Ethics Commission in the Use of Animals. Protocol ID: CEUA/IBUSP 201/2014.

Transferring the mdx mutation to the 129/Sv phenotype

The first breeding pairs consisted of 129/Sv males and mdx females. Their offspring (generation \( mdx^{129} \) F1) consisted of affected males and carrier females. The carrier females were backcrossed with the 129/Sv males, and their offspring (generation \( mdx^{129} \) F2) consisted of wild-type males and females, affected males and carrier females, according to Mendelian proportions. From this generation on, the litters were genotyped for the mdx mutation to select only the affected males and carrier females. These carrier females were then backcrossed with the 129/Sv male, generating \( mdx^{129} \) F3, genetically similar to F2.

Genotyping

DNA was extracted from a 0.5cm piece of tail using Proteinase K (Promega) as described (Zangala, 2007). The genotyping is done by PCR, using specific primers for the exon 23 of the murine dystrophin gene, and the product was applied in 10% acrylamide gel where different band patterns can be identified for wild type, heterozygous and affected animals, according to a previously described protocol (Shin et al., 2011).

Functional evaluations

All \( mdx^{129} \) male mice obtained in the three generations were monthly evaluated during the period of 6 months, using \( mdx^{C57BL} \) mice as controls. The number of studied male animals were: F1=9, F2=13, F3= 14.

The used tests were previously described (Chiavegatto et al., 2000) and validated in our colonies of neuromuscular disease mice models. In addition, the same researcher performed the analysis in all mice, avoiding intra-personal variability. Briefly:

a) The animals capacity of hanging from a bar by their fore limbs and by all four limbs – the animal is positioned hanging from a 3mm metal bar by its fore limbs or by all four limbs and the time it keeps hanging is counted. We consider 60 seconds to be the
maximum time and the test is successively repeated for three times, after which we calculate the average.

b) Fore limbs and hind limbs grip strength – the animal is positioned so that it will grip the grid attached to a dynamometer with its fore limbs or with its hind limbs and then is pulled by the tail until it releases the grip. This procedure is successively repeated for 5 times and the mean is calculated.

**Histological and immunohistochemical analyses**

For the histological analyses, three male animals from each group (mdx, F1, F2 and F3), were used, all in the ages of 6 months. After the animals were euthanized, the muscles of the posterior portion of the leg were dissected, fixed in cork blocks with Tissutec OCT (Qiagen), cryoprotected with talc and frozen in liquid nitrogen. The samples were cut in 6µm sections, which were then stained with hematoxylin and eosin. The slides were examined and photographed using a Zeiss AxioImager.Z1 microscope.

For histopathology evaluation in Hematoxilin/eosin (HE) staining, the following parameters were used: percentage of centronucleated fibers, fibrosis (evaluated using the quantification of picrossirius staining), regenerating fibers were quantified using immunofluorescence staining for developmental myosin (Vector). For inflammation analysis, we react muscle section with antibody for CD11b (BD Pharmingen).

For the fiber’s quantifications, fiber numbers from, at least, five different fields in the cross-sections from each animal were measured, and the total number of positive fibers were compared to the total number of counted fibers. The number of counted fibers were in a total of 600 to 2000 fibers for each animal.

**RNA purification and hybridization to arrays**

For transcriptome analysis, 3 animals from each strain were used, at the age of 6 months. Frozen muscles were finely powdered using a mortar and total RNA was extracted using RNeasy Microarray Tissue Mini Kit (Qiagen) according to manufacturer instructions. RNA contamination by DNA was verified in 1% agarose gel. Samples for hybridization were prepared using Ambion® WT Expression and GeneChip® WT Terminal Labeling (Invitrogen) and hybridized in GeneChip® Mouse Gene 1.0 ST Array (Affymetrix) chips, all according to instructions provided by the manufacturer.

**Microarray data analysis**

Pre analysis and data normalization were performed in the Expression Console™ (Affymetrix) software using the RMA (Robust Multi-array Average) algorithm. Normalized data were uploaded to the MeV software where the differentially expressed genes (DEGs) were determined by the SAM algorithm. To study the functional networks among the identified DEGs, we used the IPA software (Ingenuity® Systems,
Gene ontology function enrichment analysis was performed with the DAVID tool (Database for Annotation, Visualization and Integrate Discovery HTTP://david.abcc.ncifcrf.gov/).

**Statistical Analysis**

The functional evaluations and histological quantification were statistically analyzed using Mann-Whitney non parametric test for small samples, with Minitab software (Minitab Inc.) and p<0.05 was considered statistically significant.

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**Competing interests statement:** All experiments were approved by the Research Ethics Committee of the Biosciences Institute, University of São Paulo, protocol 201/214. This work has no conflict of interest.

**Author contribution:** PCC and MV conceived and designed the experiments. PCC, CFA, ALFS, AFRJ, DAG and SAF performed experiments. POO, CFA contributed reagents/materials/analysis tools. MV and PCC wrote the manuscript.

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TRANSLATIONAL IMPACT (394 words)

(1) **Clinical issue:** Muscular dystrophies are among the most common human genetic disorders, but there are few treatment options available. The *mdx* mouse is the most widely used animal model for DMD, presenting the same molecular and protein defect. Nevertheless, this mouse is not useful for clinical trials, because it shows a very mild phenotype. Aiming to create a model for DMD with a severe human-like functional course, and because some disease models showed a more severe phenotype in the 129/Sv background, we transferred the *mdx* mutation to the 129/Sv strain. Unexpectedly, functional analysis of the first three generations of *mdx*<sup>129</sup> showed a progressive amelioration of the phenotype, with fewer histopathological alterations and more regenerating fibers than the original *mdx*<sup>C57BL</sup>. To try to identify the factors that are protecting the new model from the dystrophic characteristics, we therefore performed a transcriptome comparative analysis of these two *mdx* strains.

(2) **Results:** The *mdx*<sup>C57BL</sup> presents three times more differentially expressed genes (DEGs) than the *mdx*<sup>129</sup>, when compared to the respective wild-type backgrounds (371 and 137, respectively). However, both models presented more super expressed genes than under expressed, indicating that the dystrophic and regenerative alterations are more associated with the activation rather than the repression of genes. Distributed in functional categories, the DEGs of both *mdx* models showed a predominance of genes of the immune system. Excluding this category, the *mdx*<sup>129</sup> model showed a decrease in participation of the endo/exocytic pathway (vesicle trafficking) and homeostasis categories, and an increase in participation of the extracellular matrix and enzymatic activity categories. The most significant DEGs exclusively expressed in the *mdx*<sup>129</sup>, were the up regulated *Spp1* and *Il1rn* genes. *SPP1* is an already known DMD prognostic biomarker, and our data are indicating that its up regulation can benefit the phenotype. The *Il1rn* gene encodes an interleukin 1 antagonist, and the inhibition of its pro-inflammatory and pro fibrotic proprieties could act protecting the *mdx*<sup>129</sup> animals from the inflammatory process. Finally, the comparison of the generations of *mdx*<sup>129</sup> showed that the effects of the background change are present since the first generation, suggesting a fast effect of the modifications.

(3) **Implications and future directions:** Our results may have important applications for forthcoming therapy in patients with muscular dystrophies, since the modulation of the expression of these differentially expressed genes in the two *mdx* strains could provide possible therapeutic targets for the dystrophic process.
References


Legend for figures

Figure 1: Graphical representation of comparative functional test in the mdx$^{C57Bl}$ as compared to the three mdx$^{129}$ generations. Data of the fore limbs in the bar test and grip strength test are shown with the median expression and standard error range for each age/strain. The number of tested animals in each generation is also presented. * p<0.05.
Figure 2: Representative comparative histological and immunohistochemical analysis of *mdx*\textsuperscript{C57Bl} and the 3 generations of the *mdx*\textsuperscript{129} in the ages of 6 months. HE staining, immunofluorescence staining for developmental myosin and CD11b, and staining of connective tissue with picrossirius. The scale bar shows 100µm.
Figure 3: Graphic representation of the distribution in functional categories of (A) total identified DEGs in the comparisons C57BL x mdx\textsuperscript{C57BL} and 129/Sv x mdx\textsuperscript{129}F3; (B) categories excluding the immune system.
Figure 4. Venn diagram showing genes in common and exclusive DEGs in each of the compared lists. (to be removed?)
Figure 5: Similar pathways are generated by the DEGs identified in the comparisons C57BL x mdx$^{C57BL}$ and 129/Sv x mdx$^{129}$, but with a different number of involved molecules.
Table 1: Histological data analyzed in three mice from each group, at the age of six months.

For fibers quantification, the fiber number from five different fields (in a total of 600 to 2000 fibers) were counted for each animal (approximating 90 to 100% of the cross-section).

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<th>% picrossirus + connective tissue</th>
<th>Regeneration % develop. Myosin + fibers</th>
<th>Inflammation % of cd 11b + fibers</th>
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<td>10,0 **</td>
<td>2,7 **</td>
<td>7,1</td>
</tr>
<tr>
<td>mdx&lt;sup&gt;129F3&lt;/sup&gt; 10</td>
<td>100</td>
<td>10,9</td>
<td>2,3%</td>
<td>4,4</td>
</tr>
<tr>
<td>mdx&lt;sup&gt;129F3&lt;/sup&gt; 11</td>
<td>100</td>
<td>13,9</td>
<td>3,6%</td>
<td>1,6</td>
</tr>
<tr>
<td>mdx^{129} F3</td>
<td>20</td>
<td>100</td>
<td>11.7</td>
<td>3.5%</td>
</tr>
<tr>
<td>-------------</td>
<td>----</td>
<td>-----</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>mean</td>
<td></td>
<td></td>
<td>12.09**</td>
<td>3.2**</td>
</tr>
</tbody>
</table>

* comparison of each group with mdx, *p<0.05, **p<0.001
Table 2: Comparative transcriptome analysis showing the number of identified differentially expressed genes (DEGs), and the proportion of up and down regulated genes in each comparison. The analysis was done in the total transcripts, and also using a filter selecting genes expressed in the muscle.

<table>
<thead>
<tr>
<th>Tested groups</th>
<th>Total DEGs</th>
<th>Skeletal muscle filter DEGs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL x 129/Sv</td>
<td>44</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>↑ 11</td>
<td>↑ 1</td>
</tr>
<tr>
<td></td>
<td>↓ 33</td>
<td>↓ 12</td>
</tr>
<tr>
<td>C57BL x mdx</td>
<td>371</td>
<td>135</td>
</tr>
<tr>
<td>C57BL</td>
<td>↑ 320</td>
<td>↑ 107</td>
</tr>
<tr>
<td></td>
<td>↓ 51</td>
<td>↓ 28</td>
</tr>
<tr>
<td>129/Sv x mdx F3</td>
<td>137</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>↑ 130</td>
<td>↑ 58</td>
</tr>
<tr>
<td></td>
<td>↓ 7</td>
<td>↓ 1</td>
</tr>
<tr>
<td>mdx C57BL x mdx F3</td>
<td>36</td>
<td>14</td>
</tr>
<tr>
<td>mdx C57BL</td>
<td>↑ 19</td>
<td>↑ 7</td>
</tr>
<tr>
<td></td>
<td>↓ 14</td>
<td>↓ 7</td>
</tr>
<tr>
<td>mdx F1 x mdx F2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>↓ 3</td>
<td>↓ 1</td>
</tr>
<tr>
<td>mdx F1 x mdx F3</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>↑ 1</td>
<td>↑ 1</td>
</tr>
<tr>
<td></td>
<td>↓ 4</td>
<td>↓ 1</td>
</tr>
<tr>
<td>mdx F2 x mdx F3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3: List of DEGs exclusive of the mdx<sup>129</sup> comparing 129/sv x mdx<sup>129</sup>

<table>
<thead>
<tr>
<th>Fold Change</th>
<th>gene</th>
<th>Entrez Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,417</td>
<td>Col5a2</td>
<td>collagen, type V, alpha 2</td>
</tr>
<tr>
<td>1,509</td>
<td>Maged2</td>
<td>melanoma antigen family D, 2</td>
</tr>
<tr>
<td>1,529</td>
<td>Thbs4</td>
<td>thrombospondin 4</td>
</tr>
<tr>
<td>1,591</td>
<td>Dcstamp</td>
<td>dendrocyte expressed seven transmembrane protein</td>
</tr>
<tr>
<td>1,634</td>
<td>Hist2h3a</td>
<td>histone cluster 2, H3a</td>
</tr>
<tr>
<td>1,726</td>
<td>Il2rg</td>
<td>interleukin 2 receptor, gamma prolyl 4-hydroxylase, alpha polypeptide III</td>
</tr>
<tr>
<td>1,783</td>
<td>P4ha3</td>
<td>topoisomerase (DNA) II alpha 170kDa</td>
</tr>
<tr>
<td>1,896</td>
<td>Top2a</td>
<td>interleukin 1 receptor antagonista</td>
</tr>
<tr>
<td>1,974</td>
<td>Il1rn</td>
<td>tenascin C</td>
</tr>
<tr>
<td>2,228</td>
<td>Tnc</td>
<td>Pleckstrin</td>
</tr>
<tr>
<td>2,423</td>
<td>Plek</td>
<td>secreted phosphoprotein 1</td>
</tr>
</tbody>
</table>

Table 3: List of DEGs exclusive of the mdx<sup>129</sup> comparing mdx<sup>C57BL</sup> x mdx<sup>129</sup> strains

<table>
<thead>
<tr>
<th>Fold Change</th>
<th>gene</th>
<th>Entrez Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3,621</td>
<td>Mup1 (includes others)</td>
<td>major urinary protein 1</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>C-type lectin domain family 4, member M</td>
</tr>
<tr>
<td>2,461</td>
<td>Clec4m</td>
<td>major histocompatibility complex, class I, A</td>
</tr>
<tr>
<td>-1,360</td>
<td>Hla-a</td>
<td>D site of albumin promoter (albumin D-box) binding protein</td>
</tr>
<tr>
<td>-1,280</td>
<td>Dbp</td>
<td>neurexophilin and PC-esterase domain family, member 4</td>
</tr>
<tr>
<td>-1,207</td>
<td>Nxpe4</td>
<td>heat shock 70kDa protein 8</td>
</tr>
<tr>
<td>-1,154</td>
<td>Hspa8</td>
<td>RIKEN cDNA 5330426P16 gene</td>
</tr>
<tr>
<td>-1,099</td>
<td>5330426p16rik</td>
<td>Rho-related BTB domain containing 3</td>
</tr>
<tr>
<td>1,132</td>
<td>Rhohtb3</td>
<td>protein phosphatase 1, regulatory subunit 3C</td>
</tr>
<tr>
<td>1,650</td>
<td>Ppp1r3c</td>
<td>establishment of sister chromatid cohesion N-acetyltransferase 1</td>
</tr>
<tr>
<td>1,803</td>
<td>Escol</td>
<td>kallikrein-related peptidase 3</td>
</tr>
<tr>
<td>1,837</td>
<td>Ifi202b</td>
<td>interferon activated gene 202B</td>
</tr>
<tr>
<td>3,547</td>
<td>Klk3</td>
<td>kallikrein-related peptidase 3</td>
</tr>
</tbody>
</table>