Title: Predictive markers of clinical outcome in the GRMD dog model of Duchenne Muscular Dystrophy

Running title: Predictive markers in GRMD dogs

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Abstract:
In the translational process of developing innovative therapies for DMD (Duchenne muscular dystrophy), the last pre-clinical validation step is often carried out in the most relevant animal model of this human disease namely the GRMD (Golden retriever muscular dystrophy) dog. GRMD dogs mimic the human disease, DMD, in many aspects including the inter-individual heterogeneity. This last point can be seen as a drawback for an animal model but is inherently related to its close resemblance to DMD patients. In order to improve the management of this inter-individual heterogeneity we have screened a combination of biomarkers in 61 two month-old GRMD dogs at the onset of the disease and *a posteriori* we addressed their predictive value on the severity of the disease. Three non-invasive biomarkers obtained at early stages of the disease were found to be highly predictive for the loss of ambulation before 6 months of age. An elevation in the number of circulating CD4+CD49dHi T-lymphocytes, and a decreased stride frequency resulting in a reduced spontaneous speed were found to be strongly associated with the severe clinical form of the disease. These factors can be used as predictive tests to screen dogs to separate them into groups with slow or fast disease progression before their inclusion into a therapeutic pre-clinical trial and therefore improve the reliability and translational value of the trials carried out on this invaluable large animal model. These same biomarkers have also been described to be predictive for the time to loss of ambulation in DMD boys, strengthening the relevance of GRMD dogs as pre-clinical models of this devastating muscle disease.
Introduction

Duchenne muscular dystrophy (DMD) is the most common genetic muscular dystrophy, affecting 1 in 3,500-5000 male births. It is caused by mutations in the dystrophin gene, leading to functional loss or absence of the protein at the sarcolemma of muscle fibers (Deconinck and Dan, 2007). DMD patients display progressive muscle weakness leading to the permanent use of a wheelchair in young adolescents, and to respiratory and heart failure in young adults. The disease course, though following these constant patterns, is highly variable between patients, a striking example being the age for the loss of ambulation which can range from 6 to 15 years (Flanigan et al., 2012, Ricotti et al., 2013). Several recent studies have focused on the phenotypic variability and have tried to define stratification criteria (Desguerre et al., 2009a, Humbertclaude et al., 2012), and identify modulators of disease severity (Flanigan et al., 2012, Pegoraro et al., 2011), in order to ultimately increase the success of clinical trials aiming to provide a therapeutic solution to treat or alleviate DMD.

In order to develop new strategies of treatment, different animal models have been used. In this context, the dystrophin-deficient dog, notably the Golden Retriever Muscular Dystrophy (GRMD) dog, represents a “translational bridge between mice and humans” (Duan, 2011), since it mimics more closely the human disease than other existing mammalian models of dystrophin deficiency (Valentine et al., 1988). GRMD dogs harbor a mutation in the dystrophin gene, and display dystrophic muscle lesions, inflammatory foci, progressive fibrosis and fatty infiltration, early locomotor impairment as well as premature death due to respiratory or cardiac failure. A wide inter-individual variability also figures among the numerous similarities shared by canine and human diseases. At first glance this inter-individual variability can be seen as a major obstacle in the evaluation of therapies at the pre-clinical stage (Banks and Chamberlain, 2008, Willmann et al., 2009), whereas this model may also be relevant to discover modulatory factors and modifier genes for this disease. Both
concerns can be addressed concomitantly: identifying predictive markers of the clinical evolution may help to increase the robustness of pre-clinical trials on the one hand, and unveil mechanisms underlying variations in disease expression on the other hand.

Using this approach, in a 74 DMD patients cohort, we have shown that the relative number of circulating T cells with a higher membrane expression level of CD49d (T+/CD49dhigh)- the α4 chain of the integrin VLA-4 - correlate with the progression and the prognosis of the disease (Pinto-Mariz et al., 2014, submitted). This integrin has been shown to be highly expressed in DMD patients, and more importantly these T-cell subpopulations have an enhanced migration potential (Pinto-Mariz et al. 2010) and participate to the damaging inflammatory infiltrate in DMD muscles (Pinto-Mariz et al., 2014, submitted). Consequently, CD49d can be used in DMD patients not only as a muscle inflammation biomarker reflecting the progression of the disease, but can also serve as a predictive biomarker (Pinto-Mariz et al., 2014, submitted). Another study has shown that timed motor performances (time to walk 30 feet) in DMD patients were also accurate to predict the time before loss of ambulation (McDonald et al., 1995).

In GRMD dogs, no such predictive marker has been identified. In this study we have assessed the predictive value of the blood lymphocyte subpopulations expressing high levels of CD49d, and of gait abnormalities at the onset of the disease. We were successful in identifying the first predictive biomarkers in GRMD and we provide a set of 3 ready-to-use tests which should significantly improve the quality of the therapeutic trials carried out in GRMD dogs.
Results

*Loss of ambulation affects one-third of GRMD dogs, and occurs before 6 months of age*

Inter-individual heterogeneity has been widely described in GRMD dogs but few clinical stratifications have been proposed (Ambrosio et al., 2009). In our hands the loss or maintenance of ambulation is the only clear dichotomic parameter that can define subgroups. This loss of ambulation occurs in 1 out of 3 GRMD dogs, in 85% of cases before the age of 6 months (fig 1). These animals develop severe contractures and an accelerated progression of their gait impairment (Barthelemy et al., 2011) leading to the loss of ambulation and permanent recumbence by 6 months of age. We defined these particularly devastating clinical presentations (i.e. loss of ambulation before the age of 6 months) as the ‘severe form’. In contrast, the remaining GRMD dogs are usually able to maintain ambulation until their death and have a ‘moderate form’ of the disease (i.e., dogs with either a loss of ambulation after the age of 6 months or no loss of ambulation).

The aim of the present study is to assess if blood and gait parameters could predict the evolution towards one of these two clinical forms, at an early stage of the disease. The age of 2 months was targeted, because this is the age of weaning for the puppies and is also considered as the clinical onset of the disease. From a practical point of view it corresponds to the earliest time-point for the longitudinal studies performed in GRMD dogs (Barthelemy et al., 2011, Thibaud et al., 2012), and a time after which most of the pre-clinical trials are designed to begin.

A total of 61 two month-old GRMD dogs were enrolled in the study, 15 of which evolved towards the severe form. Thirty five dogs were enrolled in the blood study (severe forms n=9/35), and 57 dogs in the motor study (severe forms n=15/57). The proportion of the severe form in each group was consistent with the usual observations which have been made in this colony. Two dogs lost ambulation after 6 months of age (at respectively 7.33 and 7.37 months
of age), and were thus categorised as moderate forms. Their late loss of ambulation was taken into account in the survival analysis.

The proportion of CD4+ T-lymphocytes with a CD49dhi surface antigen profile is increased in 2 month old GRMD dogs with a severe clinical form

In a first set of experiments, we evaluated the membrane expression of CD49d (the α4 chain of the VLA-4), comparing the two different groups of GRMD dogs with healthy controls at 2 months of age. We found a significantly higher relative number of CD4 T-lymphocytes expressing high levels of CD49d (CD4+CD49dhi T lymphocytes) in the blood of dogs with the severe form (SF) of the disease. Otherwise, no significant differences were observed in the relative numbers of CD4+CD49dhi T lymphocytes between the dogs with moderate forms (MF) and healthy controls, nor between the relative numbers of CD8+CD49dhi T lymphocytes in the blood of GRMD and control dogs. In conclusion, a high proportion of circulating CD4+CD49dhi T lymphocytes is an early biomarker of the severe form of GRMD. The detailed results are given in Table 1, and a graphic representation is provided in figure 2.

Early gait abnormalities are more pronounced in GRMD dogs with a severe clinical form

During the gait test at 2 months of age, 23 dogs spontaneously selected to run at a trot, and 33 dogs to run at a bound gallop, while 1 dog walked. Seventy percent of dogs (n=30/43) which would develop the moderate form ran at a bound gallop, while only 20% of dogs (n=3/15) which would develop the severe form used this gait type. Therefore the spontaneous use of the bound gallop at the age of two months can be considered as a good prognosis factor (χ² = 12.48, p = 0.0019).
Consistent with these gait differences, the dogs which would develop the severe form also ran significantly slower than dogs who would develop the moderate form (p < 0.0001), mainly due to a significantly lower stride frequency (p < 0.0001) associated with a shorter stride length (p = 0.004). Accordingly, the total power was lower in dogs with the severe form (p = 0.0001), as well as the force index (p = 0.017). From a more global point of view, despite the absence of differences between dogs which would develop the moderate or severe form regarding regularity and three-axial power distribution, the gait quality index revealed that the gait was significantly more impaired in dogs with the severe form (p = 0.002), in accordance with the motor score which was significantly higher in these individuals (p = 0.003). The serum creatine kinase (CK) values were slightly decreased (p = 0.016) in the GRMD dogs which would develop the severe form of the disease, and which were already less mobile.

Detailed results are given in Table 1, and graphic representations are provided in figure 3.

*Increased proportion of CD4⁺CD49d^{hi}T cells, decreased speed, and stride frequency at 2 months of age are associated with an increased risk for early loss of ambulation*

After adjustment for body mass index, number of blood leucocytes, and presence of carpal transient contractures (a common clinical observation in young GRMD dogs that can impede locomotion, I Barthelemy, S.Blot unpublished data), the proportion of CD4⁺CD49d^{hi}T cells at two months of age was linearly and significantly associated with time to loss of ambulation (adjusted hazard ratio [aHR] of 1.28 for a 1% increase; 95% confidence interval [CI], 1.05-1.57; p=0.02).

After the same adjustment, stride frequency at two months of age was linearly and significantly associated with time to loss of ambulation (aHR of 4.76 for a 1s\(^{-1}\) decrease; 95% CI, 1.45-14.29; p<0.01).
Again after the same adjustment, speed normalized by height at withers (Speed/HW) was not linearly but significantly associated with time to loss of ambulation: dogs with a value of speed/HW of 2.5 s\(^{-1}\) and dogs with a value of speed/HW of 3 s\(^{-1}\) had an increased risk for loss of ambulation of 17.70 (95% CI, 3.57-87.80; p<0.01) and 5.28 (95% CI, 2.14-13.05; p<0.01), compared to dogs with a value of speed/HW of 4 s\(^{-1}\) (Table 2, Figure 4).

**Predictive tests at 2 months of age can be proposed using the proportion of CD4\(^+\) CD49d\(^{Hi}\), the spontaneous walking speed and the stride frequency**

In order to determine to what extent the lymphocyte and gait biomarkers could be used as predictive biomarkers for disease severity, and to assess the value of such a prognostic test, ROC (receiver operating characteristics) curve analyses were performed.

Ten markers were tested: the proportion of circulating CD4\(^+\) CD49d\(^{Hi}\) and of CD8\(^+\) CD49d\(^{Hi}\) lymphocytes, the spontaneous speed normalized by the height, the stride frequency, the motor score, the gait quality index, the total power of gait, the force index and the serum CK activity. All the ROC analyses led to statistically significant results, except for the proportion of CD8\(^+\) CD49d\(^{Hi}\). The areas under the significant ROC curves ranged from 0.683 (Serum CK) to 0.881 (Speed/HW). The graphic representation of the ROC curves is shown in figure 5, and the results of the ROC curve analyses are given in table 1.

According to the results of the ROC curve analyses, the use of these three combined markers can be proposed to predict the severe forms of GRMD as early as 8 weeks of age, each with interesting characteristics that can answer different needs of a study design. The speed/HW offers the best combination between sensitivity and specificity: by positioning a cutoff at 3.2759 s\(^{-1}\), the severe form (below this cutoff) can be predicted with a 86.7 % sensitivity and a 87.8 % specificity. The specificity of the test can be increased to lessen the risk of the prediction of false severe forms by choosing the use of the stride frequency: with a cutoff
frequency of 2.44 cycles/s stride, the severe form can be predicted with a 90.2 % specificity but only a 73.3 % sensitivity. Finally, the %CD44CD49d\textsuperscript{bl} marker can be used with a 12 % cutoff leading to a 88.9 % sensitivity and 68.0 % specificity. However, this marker is of particular interest if a 100 % specificity is required: since a 14.97 % cutoff can be selected in this case, allowing a reliable selection of dogs with a predicted loss of ambulation, despite a large, but in some cases acceptable, proportion of undetected severe forms (44 % sensitivity).

One of these three tests can be chosen, as a function of the requirements of the study design, so that it is possible to select either severe or moderate clinical phenotypes, to work on during pre-clinical trials. This should improve the analysis of the clinical outcome of treated dogs, by reducing inter-individual heterogeneity and increasing the significance of results.
Discussion

In this study we have demonstrated that a set of carefully selected biomarkers can be used to predict the clinical evolution of GRMD dogs, the reference pre-clinical model of Duchenne muscular dystrophy.

Due to the strong histological and clinical similarities shared with Duchenne muscular dystrophy patients, GRMD dogs have been shown to play a key role in the translational approach to develop candidate therapies for this disease. These canine counterparts of DMD patients represent the ideal model to assess the efficacy of therapies at biochemical, histological and functional levels, in a pathological context very close to the human situation (Banks and Chamberlain, 2008, Bartlett et al., 1996).

The present study demonstrates that by using these non-invasive biomarkers obtained at early stages of the disease GRMD dogs can be separated into groups with slow or fast disease progression before their inclusion into a therapeutic pre-clinical trial thus reinforcing the translational value of this animal model.

*Predictive biomarkers will improve the translational power of pre-clinical studies carried out on GRMD dogs.*

The novel predictive biomarkers we have identified offer the opportunity for a better handling of inter-individual heterogeneity, a prominent feature of GRMD dogs. Indeed GRMD dogs are such a faithful model of DMD that they also reproduce the clinical heterogeneity seen in the human disease. However, in the context of an animal model, this can be seen as a major drawback when evaluating the effect of treatments, especially when the results are obtained in order to be directly translated to humans (Banks and Chamberlain, 2008). Recently, efforts have been undertaken to develop novel evaluation tools that allow a reliable functional
evaluation of dogs, and are able to detect the effect of a treatment, despite this clinical heterogeneity (Barthelemy et al., 2012, Marsh et al., 2010, Thibaud et al., 2012). As a supplementary tool, the knowledge of the clinical status of the dogs at the beginning of the studies (i.e. possibility to divide dogs into groups with severe and moderate phenotype) would greatly increase the significance of the results and their translational value. One can even imagine selecting dogs upon their predicted evolution as an inclusion criterion. For example, only dogs with a predicted loss of ambulation before six months of age would be included, to evaluate the effect of a given treatment to prevent the loss of ambulation. In this case, the maintenance of the ability to walk would be a positive result per se.

Moreover, such a selection of the dogs by their predicted clinical profile would lead to homogeneous GRMD dog cohorts in pre-clinical trials, and thus reduce the number of dogs required to demonstrate a therapeutic effect, in the same way as it is envisioned in clinical trials by stratifying patients upon their genotype regarding modifier genes (Bello et al., 2012). This reduction in the number of dogs should lead to faster pre-clinical results and consequently a faster initiation of clinical trials. In conclusion, these predictive biomarkers could help to accelerate the translational process of therapies for DMD.

Moreover, it should be noted that the biomarkers we have presented in this study are ready to be used in predictive tests with known thresholds, sensitivities and specificities. They are reliable, easy- and fast- to obtain and non-invasive (blood sample and gait test). Their use before the initiation of a potential treatment is thus a realistic proposal.

*These predictive biomarkers common to DMD patients strengthen the translational value of the GRMD model*
Another remarkable highlight of this study is the reinforcement of the already known similarities which exist between DMD patients and the GRMD model. First, in a previous study we have demonstrated that the proportion of circulating lymphocytes expressing high levels of CD49d is correlated to the severity of the phenotype in DMD patients, and is able to reliably predict the age at which ambulation is lost (Pinto-Mariz et al., 2014, submitted). In this study we report the same finding in GRMD dogs, at least for the CD4^+ population. Secondly, the early gait abnormality which we have described is also very close to what has been described in DMD patients. Indeed, it has been shown that the time to walk 30 feet, i.e. the spontaneous speed of gait, was strongly predictive of the time at which ambulation was lost (McDonald et al., 1995). Therefore, in both DMD patients and GRMD dogs, the speed of walking can be used to predict the loss of ambulation.

The fact that both DMD patients and GRMD dogs share the same predictive biomarkers is a new clue of the similarities existing between these two diseases and it emphasizes the interest of the GRMD model in translational research. Thus, GRMD dogs are not only similar to DMD patients from many points of view including clinical heterogeneity, but also their management during therapeutic trials can be very close to what is done for DMD patients: similar evaluation tools have been developed for both species and this is now supported by reliable predictive biomarkers. Therapies targeting DMD can thus be pre-clinically tested in the same pathological and clinical context, using the same evaluation tools and the same biomarkers as controls of the “should-be” clinical situation, optimizing the translation of results from the pre-clinical model to DMD patients.

**Limitations**

Despite the step forward in pre-clinical trials for DMD which is provided by the identification of these new predictive biomarkers in GRMD dogs, this study also presents some limitations.
First, because of the small number of dogs that were co-tested it was not possible to test the interaction that exists between lymphocytes and gait biomarkers. This should of course be done in future experiments, in order to propose a combined predictive test with an improved robustness, and also to better understand how these two features may interact. Indeed even if gait modifications are complicated to decipher, and to link to a particular pathogenic pathway, since they result from a very global functional evaluation, they could originate from an enhanced muscle inflammation in severely affected animals, as suggested by the circulating lymphocyte biomarker. A more active inflammatory process in severely affected dogs, at least partly due to CD49d\textsuperscript{hi} expressing T-cell migration to muscles, may negatively impact ambulation either directly by inducing muscle pain, or by leading to increased muscle fibrosis, as described in patients with more severe clinical presentations (Desguerre et al. 2009b). This aspect still remains to be investigated in GRMD dogs, using either muscle imaging and/or biopsies.

Furthermore the objective of this work, which has been achieved, was to provide tools to improve the handling of GRMD dogs, but these biomarkers now have to be better understood from a mechanistic point of view. Further experiments will have to be carried out to explain the early modification of these biomarkers in dogs with severe forms of the disease. In DMD patients, it has been shown that a larger number of CD4\textsuperscript{+} and CD8\textsuperscript{+} CD49d\textsuperscript{hi} circulating cells, are present in severely affected patients, these cells have an enhanced migration capacity and are also a significant component of the lymphocytic population present in the muscles, where they probably contribute to the deleterious inflammatory process (Pinto-Mariz et al., 2014, submitted). Preliminary data obtained on GRMD muscle biopsies show that CD4\textsuperscript{+}CD49d\textsuperscript{+} cells can be found in the inflammatory infiltrate like in DMD patients, but a correlation to the level of circulating TCD4+CD49d\textsuperscript{hi} cells remains to be assessed. A characterization of the inflammatory process in GRMD muscles will have to be performed in the future and
compared to what is known in DMD, to explain some differences with the human context notably why the expression of CD49d on CD8+ cells is not modified in the canine species.

*These predictive biomarkers could help to validate modulatory pathways of both the canine and human diseases*

In response to these limitations, further experiments should focus on the mechanisms which underlie the modifications of these biomarkers in severely affected dogs and humans. In this context the GRMD dog is a favorable model to study inter-individual heterogeneity, because all the dogs share the same mutation in the dystrophin gene, the same environmental conditions, and the same clinical management. It is thus much easier to work on modulatory pathways of the disease in dogs than in humans. Further experiments, including in vitro migration assays, and in vivo pharmacological blocking of CD49d, will maybe confirm the CD49d-driven inflammation hypothesis. This canine cohort should also be genotyped for genetic modifiers of the human disease severity (Flanigan et al., 2012, Pegoraro et al., 2011) in order to determine to what extent the canine situation is comparable to the human one. This genetic investigation would also represent the opportunity to study the potential link between the CD49dhi T-cells, SPP1 and LTBP4 biomarkers in the same individuals. This could help to confirm the presumed role of the TGFβ pathway in the modulation of the disease severity (Bartolome et al., 2003, Flanigan et al., 2012), or to link these biomarkers to other pathogenic pathways, paving the way for new therapeutic targets translatable from GRMD to DMD.

**Conclusion**

This study, carried out on the canine pre-clinical model of DMD, has taken advantage of its well-known inter-individual heterogeneity, one of the numerous common features with DMD,
to identify predictive biomarkers of disease evolution. Lymphocytic and gait biomarkers, common to DMD patients, were successfully found to be able to predict severe forms of the canine disease. This study enhances the already high translational value for DMD of results obtained on GRMD dogs, by reinforcing the similarities between dogs and humans affected with muscular dystrophies, and by providing new tools to overcome the issue of inter-individual heterogeneity and to improve the quality of pre-clinical trials involving GRMD dogs. Finally, these biomarkers represent new avenues to explore, to better understand clinical heterogeneity, and an opportunity to identify major modulatory pathways in dystrophin-deficient disease.
Materials and Methods

GRMD dogs

All procedures were approved by the common ethical committee of the ANSES, ENVA and UPEC (ComEth ANSES/ENVA/UPEC), under the approval number 11/01/11-07.

The GRMD dogs included in this study were housed in the facilities of the neurobiology laboratory of the Veterinary School of Alfort. They were genotyped before the age of 2 months, as previously described (Bartlett et al., 1996). Healthy littermates matched for both gender and age were used as controls for the cytofluorometric experiments. The GRMD dogs were part of a natural history study, and a regular clinical follow-up was carried out throughout their whole life. Over a period of 4 years, a total of 61 GRMD dogs were included in the study, they were all tested at 2 months of age, and categorized at 6 months of age upon their ambulation status: ambulant at 6 months of age (moderate form) versus non-ambulant at 6 months of age (severe form). Data regarding the age at which ambulation was lost, if this event occurred, were also collected.

Blood sample

Of the 61 GRMD dogs included in the study, 35 underwent an 8 mL venous blood sample at 2 months of age and were compared to eight healthy littermates. Among these 35 GRMD dogs, 26 were categorized as moderate forms, and 9 as severe forms at 6 months of age. At the time of sampling, the dogs were checked for concurrent infections, and particularly aspiration pneumonias. A blood cell count was also performed, as well as a biochemical assessment including a serum creatine kinase (CK) activity measurement.

Cytofluorometry

For cytofluorometry we used fluorochrome-labeled monoclonal antibodies with specificities for CD3, CD4, CD8 (Serotec, Kidlington, UK) and CD49d (PharMingen/Becton-Dickinson,
San Diego, USA). Isotype/fluorochrome-matched unrelated antibodies were obtained from Pharmingen/Becton-Dickinson. Peripheral blood mononuclear cells (PBMCs) from GRMD and healthy controls were isolated through ficoll-histopaque (Sigma-Aldrich, St Louis, MO, USA) sedimentation, using freshly obtained samples. PBMC were first incubated in 96 well plates -- with 5% fetal calf serum for 20 minutes at 4°C -- and then subjected to fluorochrome-labeled primary monoclonal antibodies for 30 minutes. After washing, cells were fixed and acquisition for flow cytometry was carried out using a LSR II® flow cytometer (Becton Dickinson, San Jose, USA) equipped with FacsDiva software. A cell gate excluding cell debris and non-viable cells was determined using forward versus side scatter parameters. Analyses were done after recording 20,000 events for each sample, using the ModFit LT software.

Functional motor assessment

Among the 61 GRMD dogs included in the study, fifty-seven 2 month-old GRMD dogs underwent a locomotor evaluation encompassing a clinical motor score, as well as a 3D-accelerometry test. Among them, 15 were then classified as severe forms due to a loss of ambulation before 6 months of age.

The clinical motor score was performed using a previously described scoring grid (Barthelemy et al., 2012, Thibaud et al., 2007), containing 11 items; the higher the score was, the more severe was the motor phenotype. The motor score was expressed as a percentage of the maximal score.

The 3D-accelerometry test was performed as previously described using a dedicated device (locometrix®, Centaure Metrix, Evry, France) intending to record three-axial accelerations near the center of gravity during spontaneous gait (Barthelemy et al., 2009). Ten previously described variables were calculated from the acceleration curves in the software Equimetrix®
and are as follows: the speed (m/s), the stride frequency (cycles/s), the stride length (m), the regularity, the dorso-ventral, cranio-caudal and medio-lateral powers (W/kg), the total power (W/kg), the force index (expressed in N/kg body weight, and calculated by normalizing the total power (W/kg) by the speed (m/s)), and a gait quality index recapitulating the association of 7 variables. The speed and stride length, because directly influenced by the dog size, were normalized by the height at the withers (HW, m), and the axial powers were expressed as a proportion of the total power (%).

**Statistical analyses**

Univariate comparisons between severe and moderate forms were assessed using chi-square or Student t-tests for categorical (eg the gait type) or continuously distributed variables, respectively.

For each biomarker that was associated with loss of ambulation at 6 months (ie, severe versus moderate form) with a P-Value < 0.05, a receiver operating characteristics (ROC) curve was used to evaluate the effectiveness of the biomarker for distinguishing dogs with severe form from those with moderate form.

Survival analyses were performed to assess the association between time to loss of ambulation and three following exposure variables: 1/ proportion of CD4⁺CD49d⁺ T lymphocytes, 2/ stride frequency, or 3/ speed normalized by the height. Univariate and multivariate Cox proportional hazard models were used for each of the three exposure variables. The candidates for potential confounding variables were: body mass (kg), body mass index (BMI, calculated by dividing the body mass (kg) by the height at withers (m²)), presence of a carpal contracture, and blood leucocytes number. These last three candidates were associated with loss of ambulation with a P-Value < 0.20 in univariate analyses and were therefore included into each multivariate Cox model.
Spline functions (Desquilbet and Mariotti, 2010) were used to check the linearity assumption for each of the three exposure variables; this assumption was valid (i.e., p-value for a non-linear association > 0.30) for proportion of CD4+CD49d^high T lymphocytes and stride frequency, but not for speed normalized by the height (p-value for a non-linear association < 0.01). In the latter model, a spline function with three knots located at 5th, 50th, and 95th percentiles was used for speed normalized by the height. For optimal adjustments, BMI as well as a leucocytes were both included using spline functions with 3 knots located a 5th, 50th, and 95th percentiles (Desquilbet and Mariotti, 2010).

Analyses were conducted using Statistica (version 10, Stat Soft, Maisons-Alfort, France) and SAS® V9.2 (SAS Institute, Cary NC) softwares. The level of significance was set at 0.05.

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Competing interests:

We have no competing interest to declare.

Author contributions:

IB, FPM, WS, SDSB, VM, TV, SB and GBB designed the experiments, IB, FPM, EY, AMF performed the experiments, IB, FPM, LD analyzed the data, IB, FPM, LD, TV, SB and GBB wrote the paper.
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References


Figure legends

Fig. 1. Loss of ambulation in GRMD dogs and classification in two clinical forms as a function of the occurrence of this clinical event.

On the left panel, the curve represents a Kaplan-Meier curve that shows the percentage of ambulant dogs according to age obtained on 94 GRMD dogs (S. Blot, historical data). Loss of ambulation events are represented in red, and censored data (ambulant dogs) in green. The loss of ambulation occurs in about one third of the dogs, mainly before 6 months of age (85% of the dogs with a loss of ambulation). A classification into two sub-phenotypes is proposed and illustrated on the right panel: severe form (red frame) are opposed to moderate form (green frame) and are characterized by a loss of ambulation before 6 months of age and an early death (euthanasia due to the permanent recumbence).

Fig. 2. Analysis of the expression of CD49d on circulating T lymphocytes, and results in 2 month-old dogs as a function of their clinical form.

A and B respectively show typical CD49d cytometric profiles from a moderate form (A, green frame) and a severe form (B, red frame). Upper profiles show the profile of CD49d expression in CD4+ cells, and lower profiles in CD8+ cells. The population expressing high levels of CD49d is located to the right of the black dotted line drawn on the graphs. The graphs C and D respectively represent the comparison of the percentages of CD4+CD49dhi and CD8+CD49dhi obtained in 2 month-old GRMD dogs with would-be moderate forms (GRMDMF, left part of the graphs, in green) versus severe forms (GRMDSF, right part of the graphs, in red). The height of the histograms provides the mean, and the error-bars indicate ±1 standard-deviation. Individual values have been superimposed to the histograms and are represented by empty circles. The symbol ** indicates a significant difference (p < 0.01), and NS means “not significant”.

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Fig. 3. Analysis of the motor function and results of the gait analysis, motor score and creatine kinase level in 2 month-old dogs as a function of their clinical form.

A and B respectively show a 10 second-sample of dorso-ventral acceleration curves from 2 month-old GRMD dogs which would develop either moderate (green) or severe (red) forms, at a trot. It should be noted that the frequency and the amplitude of the curves are lower in the case of the GRMD dogs that would develop the severe form of the disease. The graphs C to J represent the comparison of the values obtained in 2 month-old GRMD dogs which would develop the moderate form (GRMD^MF, left part of the graphs, in green) versus severe form (GRMD^SF, right part of the graphs, in red). The height of the histograms provides the mean and the error-bars indicate ± 1 standard-deviation. Individual values have been superimposed to the histograms and are represented by empty circles. Significant differences are represented by * if p < 0.05, ** if p < 0.01, and *** if p < 0.001. C: speed normalized by the height at withers (HW); D: stride frequency; E: stride length normalized by the height at withers (HW); F: total power; G: force index; H: gait quality index; I: motor score; J: serum creatine kinase (CK).

Fig. 4. Association between age at which ambulation was lost and speed /HW at 2 months of age using a spline function with three knots

Adjusted spline function analysis of the association between age at loss of ambulation and speed normalized by height at withers (HW), with three knots located a 5th, 50th, and 95th percentiles. This curve illustrates the very high hazard ratios at low speed /HW values. For example, dogs with a value of speed/HW of 2.5 s⁻¹ have an increased risk for loss of ambulation of 17.70 (95% CI, 3.57-87.80; p<0.01) compared to dogs with a value of speed/HW of 4 s⁻¹ (median of the whole population). The individual values of speed /HW are schematized under the graph (crosses).

Abbreviations: HW: height at withers; HR: hazard ratio; CL: confidence level

Fig. 5. ROC curves of the candidate markers for a predictive test

The ROC curves of the ten parameters tested for their ability to predict severe forms are schematized on this graph. Each marker is represented by a specific color, indicated in the caption on the right. Note that the curves with the highest areas under the curve (AUC) are those obtained using the speed (AUC = 0.881) and the stride frequency (AUC = 0.862). Another interesting point is that the percentage of CD4+CD49dHi (AUC = 0.853) remains highly specific (100 %) until relatively high levels of sensitivity (44 %). Abbreviations: HW: height at withers; GQI: gait quality index; CK: creatine kinase.

Tables:
### Table 1. Mean values of the markers at 2 months of age and statistical results.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD) Healthy dogs</th>
<th>Mean (SD) GRMD SF</th>
<th>Mean (SD) GRMD MF</th>
<th>p-value (GRMD SF vs GRMD MF)</th>
<th>Area under the ROC curve (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8⁺CD49d^- (%) total CD8⁺</td>
<td>66.29 (13.31)</td>
<td>64.34 (13.11)</td>
<td>65.81 (14.35)</td>
<td>0.8094</td>
<td>0.571 (0.595)</td>
</tr>
<tr>
<td>CD4⁺CD49d^- (%) total CD4⁺</td>
<td>10.26 (2.70)</td>
<td>15.78 (4.65)</td>
<td>10.44 (2.71)</td>
<td>0.0087</td>
<td>0.853 (0.0001)</td>
</tr>
<tr>
<td>Speed/HW (s⁻¹)</td>
<td>5.68 (0.90)</td>
<td>2.81 (0.86)</td>
<td>4.50 (1.14)</td>
<td>&lt; 0.0001</td>
<td>0.881 (0.0001)</td>
</tr>
<tr>
<td>Stride frequency (cycles/s)</td>
<td>2.89 (0.20)</td>
<td>2.41 (0.44)</td>
<td>3.15 (0.52)</td>
<td>&lt; 0.0001</td>
<td>0.862 (0.0001)</td>
</tr>
<tr>
<td>Stride length/HW</td>
<td>1.97 (0.26)</td>
<td>1.21 (0.27)</td>
<td>1.41 (0.20)</td>
<td>0.0040</td>
<td>0.753 (0.0002)</td>
</tr>
<tr>
<td>Total power (W/kg)</td>
<td>81.5 (18.6)</td>
<td>30.9 (13.9)</td>
<td>62.1 (25.9)</td>
<td>0.0001</td>
<td>0.847 (0.0001)</td>
</tr>
<tr>
<td>Force index (N/kg)</td>
<td>53.2 (6.3)</td>
<td>38.2 (15.4)</td>
<td>50.5 (16.2)</td>
<td>0.0173</td>
<td>0.704 (0.0055)</td>
</tr>
<tr>
<td>GQI</td>
<td>1.14 (0.60)</td>
<td>3.46 (0.99)</td>
<td>2.65 (0.69)</td>
<td>0.0019</td>
<td>0.793 (0.0001)</td>
</tr>
<tr>
<td>Motor score (%)</td>
<td>81.5 (18.6)</td>
<td>25.9 (6.1)</td>
<td>19.8 (6.1)</td>
<td>0.0034</td>
<td>0.779 (0.0003)</td>
</tr>
<tr>
<td>Serum CK (IU/L)</td>
<td>53.2 (6.3)</td>
<td>23092 (11623)</td>
<td>37757 (31117)</td>
<td>0.0160</td>
<td>0.683 (0.022)</td>
</tr>
</tbody>
</table>

Abbreviations: SD: standard deviation; GRMD SF: GRMD dogs affected with severe forms; GRMD MF: GRMD dogs affected with moderate forms; ROC: Receiver operating characteristic; HW: height at withers; GQI: gait quality index; CK: creatine kinase.

### Table 2. Results of the spline function analysis of the association between age at loss of ambulation and speed /HW at 2 months of age

<table>
<thead>
<tr>
<th>Speed_HW (s⁻¹)</th>
<th>aHRs</th>
<th>Lower_95CI</th>
<th>Upper_95CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>65.71**</td>
<td>6.12</td>
<td>705.88</td>
</tr>
<tr>
<td>2.5</td>
<td>17.7**</td>
<td>3.57</td>
<td>87.8</td>
</tr>
<tr>
<td>3</td>
<td>5.28**</td>
<td>2.14</td>
<td>13.05</td>
</tr>
<tr>
<td>3.5</td>
<td>1.95**</td>
<td>1.37</td>
<td>2.78</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>0.76*</td>
<td>0.6</td>
<td>0.96</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>0.47</td>
<td>1.38</td>
</tr>
<tr>
<td>5.5</td>
<td>1.08</td>
<td>0.4</td>
<td>2.93</td>
</tr>
<tr>
<td>6</td>
<td>1.68</td>
<td>0.35</td>
<td>8.09</td>
</tr>
</tbody>
</table>
Abbreviations: aHR, hazard ratio adjusted for BMI, leucocytes number, and carpal contracture; CI, confidence interval; * p-value < 0.05; ** p-value < 0.01
Translational impact

Clinical issue

Duchenne muscular dystrophy (DMD) is a genetic disease involving the whole striated and cardiac musculature and leading to loss of ambulation in young teenagers and death by the third decade. No curative solution is available yet for this disease. Canine models of DMD including GRMD (Golden retriever muscular dystrophy) closely mimic the human situation and are a relevant context to address pre-clinical questions. Among the similarities between canine and human diseases, the inter-individual clinical heterogeneity is a prominent feature, making results from pre-clinical and clinical studies difficult to decipher. In this study, motor and lymphocytic biomarkers have been evaluated at an early stage of the canine disease, and their predictive value has been assessed, in order to develop a predictive test, that could improve the translational value of pre-clinical studies.

Results

A 61 GRMD dogs population evaluated at 2 months of age (clinical onset) has been divided in 2 subgroups according to the occurrence of loss of ambulation before 6 months of age (severe form) affecting one fourth of the dogs. An elevated proportion of circulating lymphocytes expressing high levels of the integrin CD49d was found predictive of severe forms. In the same way, low spontaneous gait speed and stride frequency were strongly associated with the loss of ambulation. Interestingly these markers have also been described predictive of the time to loss of ambulation in DMD patients. The reliability of a predictive test based on these non-invasively and simply-obtained markers was assessed, demonstrating that they can be used to classify dogs upon their future clinical evolution, with good specificity and sensitivity values.

Implications and future directions
This study provides ready-to-use predictive tests that can significantly improve the quality of conclusions drawn at the pre-clinical key-step of the evaluation of candidate therapies for DMD. Indeed the use of these biomarkers can be realistically envisioned, either as inclusion criteria, or as covariates in the analysis of study results. Beyond the expected positive consequences of a pre-clinical step improvement for clinical studies, the fact that the predictive biomarkers are shared by both species reinforces the translational relevance of this canine counterpart of DMD. Indeed clinical heterogeneity could be managed in the same way during pre-clinical and clinical steps, optimizing the translation of results. Future experiments should focus on the mechanisms underlying the variation of these biomarkers in function of the clinical form, and could lead to the identification of therapeutic targets for DMD.
**CD4+**

**CD8+**

Relative number of cells

CD49d

**NS**