Two pools of inorganic phosphate in canine model of DMD caracterized by magnetization transfer ^{31}P NMRS

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Introduction: In Duchenne muscle dystrophy (DMD) and in animal models of the disease, anomalies in *in vivo* ^{31}P NMRS have been repeatedly reported[1]. Altered energetic phosphometabolite content (principally inorganic phosphate (Pi), phosphocreatine (PCr) and phosphodiesters (PDE) and modified intracellular pH regulation, could directly or indirectly reflect abnormal membrane permeability and turnover. With onset of therapeutic trials in DMD, renewed interest in disease markers prompted us to revisit these questions in the GRMD canine model of the disease, and led us to identify what appears to be a second Pi resonance[2] which we aimed to better characterize.

Materials&Methods: *Dogs:* Ten *GRMD* dogs (aged 2 - 18 months) and 10 healthy litter mate controls (CONT, aged 2 - 37 months), bred at the National Veterinary School of Alfort were investigated. During anaesthesia, (induced by propofol, and maintained with 2% isofluorane in O_2), dogs were infused with isotonic NaCl, heart rate and oxygen saturation were constantly monitored, and body temperature was maintained by a heating water bed. *NMR investigation – baseline:* Dogs were installed feet first on their sides in a 4T, 46-cm free bore, magnet interfaced to a Bruker Biospec spectrometer. An in-house 2-cm-diameter double-tuned ^{1}H-^{31}P coil was carefully placed by palpation over the tibial cranial (TC) or biceps femoris (BF) muscles, and position confirmed by fast GE imaging. Careful shimming to better than 17Hz on PCr, including 8Hz line broadening, was ensured using FASTMAP. Baseline resting ^{31}P NMRS spectra were acquired for 6-12 minutes (200 µs broad pulse, 2048 complex points, SWH 3kHz, TR 1.5s). *NMR investigation – chemical exchange.* Selective saturation of γ-ATP magnetization causes quantifiable reduction of PCr and Pi signal owing to rapid chemical exchange (respectively through the creatine kinase reaction and inverse hydrolysis of ATP). Magnetization transfer[3] measurements (MT), and saturation-recovery T1 measurements with MT (SRMT) were carried out in TC of 8 dogs (4GRMD, 4CONT) to determine the rate constant k_f of the inverse hydrolysis of ATP, and T1 of Pi. Selective saturation of γATP was obtained through low power square pulses (~30mW) of variable duration (0.9 - 8s) in both experiments, with a TR of 4.5s for the MT sequence, and large-band CHESS saturation for SRMT. Each of the MT and SRMT experiments required 1.5-2hrs acquisition, for adequate S/N. *Data processing.* Initial processing was carried out with Bruker TOPSPIN 1.5, for integral determination of PDE, PCr and ATP. Owing to the overlap between the two supposed *Pi* resonances, we developed a dedicated in-house model to fit 4 Lorentzian resonances *PCr*, *PDE* and the Pi_a *and* Pi_b in the region +6 to –1 ppm using *Excel*^{TM}. Asymptotic S.E. were calculated using SolverAid in MacroBundle v.7[4]. The performance of this tool was confronted to the algorithm AMARES deconvolution, implemented in jMRUI4. *Data analysis.* The M_z magnetization exponential decrease in MT and increase in SRMT tend to the common asymptote $M_0^{Pi}\, T_{1Pi}{}^{*}/T_{1Pi}$, and were fitted simultaneously for improved precision, with exponential constant $e^{-t/T_{1Pi}{}^{*}}$, where $T_{1Pi}{}^{*}=T_{1Pi}/\left(1+k_f T_{1Pi}\right)$.

Results Figure 1 shows the caracteristic second peak at ~0.3ppm upfield from Pi in GRMD, but also a shoulder to the Pi peak in CONT dogs.

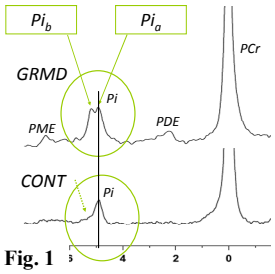

Fig. 1

Methodological validations. Processing was validated on 2 dataset series (1 CONT, 1 GRMD) of 32 spectra acquired consecutively and summed in pairs (1+32, 2+31…) to rid them of system drift. Fitting the spectra with 2 resonances at Pi improved the correlation coefficient between data and model in GRMD F=18.0, p<0.0001 but also in CONT dog; F=4.04 p= 0.012. The mean of normalized SD of intensities of Pi_a and Pi_b and of corresponding pH_a and pH_b was improved with our restricted model compared to jMRUI (0.036 vs 0.11 on CONT and 0.035 vs 0.42 on GRMD) whilst jMRUI altogether failed to identify the second resonance in 25% of cases (1/8 control, 3/8 CXMD with lower S/N). The MT and SRMT sequences were first validated by determining k_f of the creatine kinase reaction, in calves of 2 human subjects. Good adequation was obtained between experimental points and theroretical fits (R^2>0.99), and yielded T_1^{PCr} (5.0 ± 0.1 and 5.3 ± 0.1s) and k_f^{PCr} 0.52 ± 0.04 and 0.51 ± 0.04 s^{-1}, within typical literature values[5].

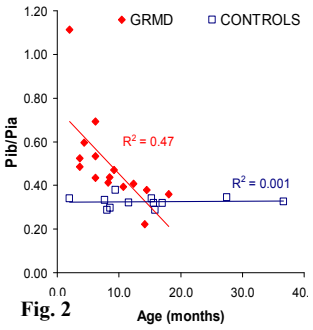

Fig. 2

Baseline ^{31}P NMRS. As expected, Pi/PCr and Pi/ATP were increased in TC and BF and PCr/ATP reduced in TC of GRMD compared to age matched CONT. Over all dogs, we determined that Pia corresponded to a physiological intracellular pHa (6.97±0.05), whilst pHb of the more alkaline peak Pib approached a more extracellular range (7.27±0.10), and found pHb and pHa (for both BF and TC together) to be correlated in GRMD (R^2=0.51) , but not in CONT (R^2=0.001). Interestingly, Pib/ Pia was highest in GRMD but diminished with age to CONT levels (Fig. 2).

Chemical exchange. MT and SRMT experiments (Fig.3) indicated that T1 relaxation times were similar for Pia and Pib compartments in GRMD and CONT, but chemical exchange was very much reduced in GRMD, compared to Pia of CONT. To be noted, Pib/Pia and Pi/ATP, but not Pi/PCr, were significantly increased in this subgroup of GRMD vs CONT.

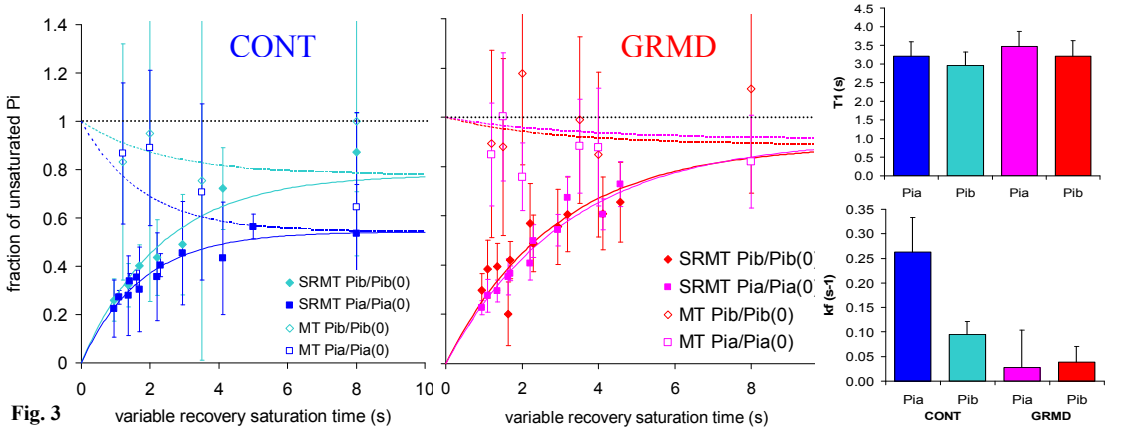

Fig. 3

Discussion The data show the existence of what may be supposed to be a more alkaline, less metabolically active Pi pool in dogs, and that this pool is considerably enhanced in young GRMD. The more acidic Pia resonance observed in GRMD most likely originates from the cytosolic Pi, because its properties (T1, pH) are similar to those of the dominating Pi peak in CONT, although the drop in k_f of Pi_a remains to be understood. Among hypotheses that can be evoked regarding the Pib resonance, a contamination by blood resonance can be excluded owing to absence of other blood metabolite contamination, such as 2,3-DPG. Mitochondrial Pi in muscle would be expected to have a different T1[6] and to be more, not less, metabolically active. Alternately, the higher pH and reduced metabolism could correspond to that of interstitial compartment, but would imply equal extracellular and intracellular volumes which is very improbable. Alternatively, in GRMD dogs, Pib could originate from suffering cells presenting a leaky membrane, with inadequate cell homeostasis and less efficient regulation of pH. However, in CONT, the presence of a low intensity second peak, whose pH is not correlated to the first, is still unexplained. Continued investigations are required to clarify the nature of this alkaline component, its relation to pathophysiological processes and its potential value as a biomarker of DMD.

1. M.A. Cole, et al., Neuromuscul Disord, 2002. 12. 247. **2.**J.L. Thibaud, et al., Acta Myologica, 2010. 29. 225. **3.**S. Forsen, et al., Journal of Chemical Physics, 1963. 39. 2892. **4.**R. De Levie.*(http://www.bowdoin.edu/~rdelevie/excellaneous/)* **5.**J.F. Goudemant, et al., Magn Reson Med, 1997. 37. 744. **6.**H.E. Kan, et al., NMR Biomed, 2010. 23. 995.