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Fibroblast growth factor 19 as a countermeasure to muscle and locomotion dysfunctions in experimental cerebral palsy

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Abstract

Background Cerebral palsy (CP) associates cerebral function damages with strong locomotor defects and premature sarcopenia. We previously showed that fibroblast growth factor 19 (FGF19) exerts hypertrophic effects on skeletal muscle and improves muscle mass and strength in mouse models with muscle atrophy. Facing the lack of therapeutics to treat locomotor dysfunctions in CP, we investigated whether FGF19 treatment could have beneficial effects in an experimental rat model of CP.

Methods Cerebral palsy was induced in male Wistar rat pups by perinatal anoxia immediately after birth and by sensorimotor restriction of hind paws maintained until Day 28. Daily subcutaneous injections with recombinant human FGF19 (0.1 mg/kg bw) were performed from Days 22 to 28. Locomotor activity and muscle strength were assessed before and after FGF19 treatment. At Day 29, motor coordination on rotarod and various musculoskeletal parameters (weight of tibia bone and of soleus and extensor digitorum longus (EDL) muscles; area of skeletal muscle fibres) were evaluated. In addition, expression of specific genes linked to human CP was measured in rat skeletal muscles.

Results Compared to controls, CP rats had reduced locomotion activity (−37.8% of distance travelled, $P < 0.05$), motor coordination (−88.9% latency of falls on rotarod, $P < 0.05$) and muscle strength (−25.1%, $P < 0.05$). These defects were associated with reduction in soleus (−51.5%, $P < 0.05$) and EDL (−42.5%, $P < 0.05$) weight, smaller area of muscle fibres, and with lower tibia weight (−38%, $P < 0.05$). In muscles from rats submitted to CP, changes in the expression levels of several genes related to muscle development and neuromuscular junctions were similar to those found in wrist muscle of children with CP (increased mRNA levels of *Igfbp5*, *Kcnn3*, *Gdf8*, and *MyH4* and decreased expression of *Myog*, *Ucp2* and *Lpl*). Compared with vehicle-treated CP rats, FGF19 administration improved locomotor activity (+53.2%, $P < 0.05$) and muscle strength (+25.7%, $P < 0.05$), and increased tibia weight (+13.8%, $P < 0.05$) and soleus and EDL muscle weight (+28.6% and +27.3%, respectively, $P < 0.05$). In addition, it reduced a number of very small fibres in both muscles ($P < 0.05$). Finally, gene expression analyses revealed that FGF19 might counteract the immature state of skeletal muscles induced by CP.

Conclusions These results demonstrate that pharmacological intervention with recombinant FGF19 could restore musculoskeletal and locomotor dysfunction in an experimental CP model, suggesting that FGF19 may represent a potential therapeutic strategy to combat the locomotor disorders associated with CP.

Keywords Fibroblast growth factor 19; Cerebral palsy; Skeletal muscle; Sarcopenia

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Introduction

Cerebral palsy (CP) is a perinatal disease affecting about 2–3 per 1,000 children worldwide.[1] CP is associated with permanent posture disorders and immobility due to neurofunctional damages of the developing brain.[1,2] Children affected by CP have robust deficiency of gait and movement and develop premature sarcopenia, with high vulnerability to weakness and increased fatigue during activities.[3] In addition, by reducing the load on the developing skeleton, the insufficient functional musculature and immobility impair the healthy development of bones.[4,5] Currently, individuals with CP are mainly treated by physiotherapy, bracing and orthopaedic surgery, which all have limited impacts for the patient's welfare.[6] Facing the reduced quality of life of these children, developing new therapeutic measures are highly warranted.[4,5]

To better understand the pathogenesis of CP and explore novel therapeutic strategies, perinatal anoxia and sensorimotor restriction of hind paws have been used to develop preclinical CP models.[7,8] In rats, this experimental CP model is characterized by reduced body growth, abnormal walking patterns, atrophy of hind limb muscles, extracellular matrix changes and joint degeneration of knee and ankle.[7,9] It is also associated with reduced locomotor activity,[10] increased spasticity,[9] impaired chewing[11] and motor skills, and reduced sarcomere density.[12] In addition, this experimental CP model shows brain alterations, such as an increase in the permeability of the blood–brain barrier[13] and a degraded representation of hind limbs in the primary motor cortex.[9]

We recently discovered that the fibroblast growth factor 19 (FGF19) increases skeletal muscle mass and strength.[14] FGF19 (and its rodent ortholog FGF15) is a member of the atypical endocrine subfamily of FGFs, produced by ileal enterocytes. In mice, treatment with recombinant human FGF19 significantly increases skeletal muscle mass and muscle fibre surface. Furthermore, FGF19 increases the size of human myotubes *in vitro*. At the signalling level, FGF19 binds to FGF receptor/ß-klotho complex and induces its hypertrophic effect by activating an extracellular-signal-regulated protein kinase 1/2 (ERK1/2)/mammalian target of rapamycin (mTOR) pathway.[14] Importantly, FGF19 treatment during 1 or 2 weeks improved muscle wasting and muscle strength in different experimental models including sarcopenic aged mice and glucocorticoid-treated mice,[14] thus supporting the therapeutic potential of FGF19 in pathologies with muscle weakness.

In the present proof-of concept study, we aimed at verifying whether FGF19 could be used as a countermeasure to fight against muscle atrophy and mobility dysfunction in a rat model of CP. We found that daily administration of human recombinant FGF19 between day 22 and day 28 after birth in CP rats, improved locomotion and musculoskeletal parameters such as muscle fibre size and tibia bone mass. In addition, FGF19 treatment restored the muscle expression of several genes that have been previously found altered in wrist muscle of children with CP.[15]

Methods

Animals

The study was approved by the Ethics Committee on Animal Use (protocol 0011/2017) and performed in accordance with the 1964 Declaration of Helsinki and its later amendments. Wistar rats were kept in the maintenance vivarium of the UFPE Department of Nutrition at a temperature of 22 ± 2 °C, inverted light–dark cycle of 12/12 h, housed in polypropylene cages with free access to water and diet. On the day of birth, male pups were randomly distributed in the experimental groups as followed: control + vehicle (V); control + FGF19 (F); CP + vehicle (CPV); CP + FGF19 (CPF). Female pups were used to complete the litter of eight pups until weaning. CP was induced by submitting male pups to two episodes of anoxia (exposure to 100% nitrogen at 9 L/min for 12 min), on the day of birth (P0) and the day after (P1). Afterwards, from P2 to P28, sensorimotor restriction of the hind limbs was performed daily for 16 h, with free movement of the animal in the remaining 8 h of the day.[7,11] Weaning occurred at P25, and after this time, the male pups were placed in individual cages. Treatment with recombinant human FGF19 (R&D System, UK) was performed from P22 to P28. All injections of vehicle solution (phosphate-saline buffer solution with 0.1% bovine serum albumin) or recombinant human FGF19 solution (0.1 mg/kg in the vehicle solution) were performed subcutaneously.[14]

Body weight and locomotor activity

Animals were weighed at P0, P8, P14, P17, P22 and P29 using an electronic digital scale (Marte, S-1000 model with 0.1 g of sensitivity). Locomotor activity was analysed at P22 and P28 in a dark room during the dark cycle when the animals are usually awake. Animals were positioned in the center of an open field and filmed (Ulead Video Studio® software) for a

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period of 5 min. Each video was analysed using the ANY-maze software to obtain the following parameters: total distance travelled (m), average speed (m/s), number of stops, and immobility time (s), as previously described.[10] Representative recordings are shown as supporting information, *Video* S1.

Motor coordination assessment

The rotarod test was performed at P29 by a blinded evaluator. One animal at a time was placed in the rotarod equipment (rod 60 mm in diameter and 75 mm in length). Five attempts were made, with a 2 min rest interval, at a speed of 25 rpm for a maximum of 3 min. The time (latency) before the fall was recorded, and the mean latency time of the five attempts was calculated (adapted from Stigger *et al*.[12]).

Muscle strength assessment

Analysis of muscle strength was performed at P22 and P28, using the suspension test (forelimb grip test), with video recording. Animal was positioned 1 m away from the ground on a coated steel cable (3 mm in diameter) and remained gripped by the forelimbs for a time limit of 60 s while suspended by the tail. Videos were analysed by a blind appraiser, using the Windows Movie Maker program, and the fall latency, expressed in seconds, was measured and the data were further expressed as arbitrary units (adapted from Teo *et al*.[16]).

Tissue sampling

At the time of euthanasia (P29), skeletal muscles [soleus and extensor digitorum longus (EDL)] and tibia bone from the hind limbs were harvested and weighted. Left posterior limb muscles were immediately frozen at −80°C for gene expression analyses. Muscles of the right hind limb were frozen in *n*-hexane (pre-cooled with dry ice) and stored at −80°C for histological analyses. The longitudinal length of the tibia bone was measured using a calliper.

Muscle fibre area measurements

To determine cross-sectional fibre size, 10 μm-thick cryosections taken at the mid-belly of the muscles (soleus and EDL) were processed for immunostaining, as described previously.[14] Briefly, sections were blocked for 1 h at room temperature and incubated overnight at 4°C with a rabbit anti-laminin antibody (Sigma, L9393), followed by incubation with a secondary antibody (AlexFluor Goat anti Rabbit IgG AlexaFluor 594—A11012 ThermoFisher). The 10× magnification images were taken using a Zeiss Axiovert200M microscope. The Axiovision software was configured to take into account only the transverse fibres with a Ferret ratio strictly up to 0.5 and their area was measured in square micrometres (μm^2).

Pax7 expression by immunohistochemistry

For Pax7 immunostaining, soleus muscle sections were first labelled with anti-Pax7 antibody (dilution at 3 μg/mL, Developmental Studies Hybridoma Bank) for 1 h, followed with AlexaFluor 555 goat anti-mouse (1:1000, Invitrogen). After washing, slides were incubated with anti-laminin antibody (1:100, Sigma Aldrich) and detected with an AlexaFluor 488 goat anti-rabbit (1:1000, Invitrogen). Then, soleus muscles were counterstained with a DAPI mounting medium (Abcam). Five to ten fields were acquired with a 20× magnification using an Olympus BX63 microscope. At least 500 fibres were used to record the $PAX7^+/DAPI^+$ satellite cells and the data were normalized by the number of laminin positive fibres.

Gene expression analysis

Total RNA from soleus and EDL muscles was extracted using TRI Reagent (Sigma Aldrich, Saint-Louis, MO, USA). RNA preparations were quantified using Nanodrop 2000 (Ozyme) and their quality was checked using Agilent bioanalyser 2100. First-strand cDNAs were synthesized from 1 μg total RNA using Prime Script RT Reagent kit (Perfect Real Time) 200X (Ozyme) and a combination of oligodT and random primers. Transcript levels were measured by real-time PCR (Rotor-Gene 6000, Qiagen, Courtaboeuf, France) in a final volume of 20 μL using the SYBR qPCR Premix Ex Taq kit (Ozyme). Each assay was performed in duplicate and validation of the RT-PCR runs was assessed by evaluating the melting temperature of the products, and by the slope and error obtained with the standard curve. The analyses were performed using Rotorgene software (Qiagen). The results were normalized to *Tbp* (TATA binding protein) expression, used as internal standard. The list of primer sequences is available in *Table* S1.

Statistics

One-way or two-way analysis of variance tests were performed to determine differences between experimental groups. *Post-hoc* comparisons were performed by Tukey's test, with statistical significance set at $P \leq 0.05$. For gene expression and immunohistochemistry, Mann–Whitney test was used. All statistics were performed using GraphPad Prism 8.4.1 and data are presented as means ± SEM.

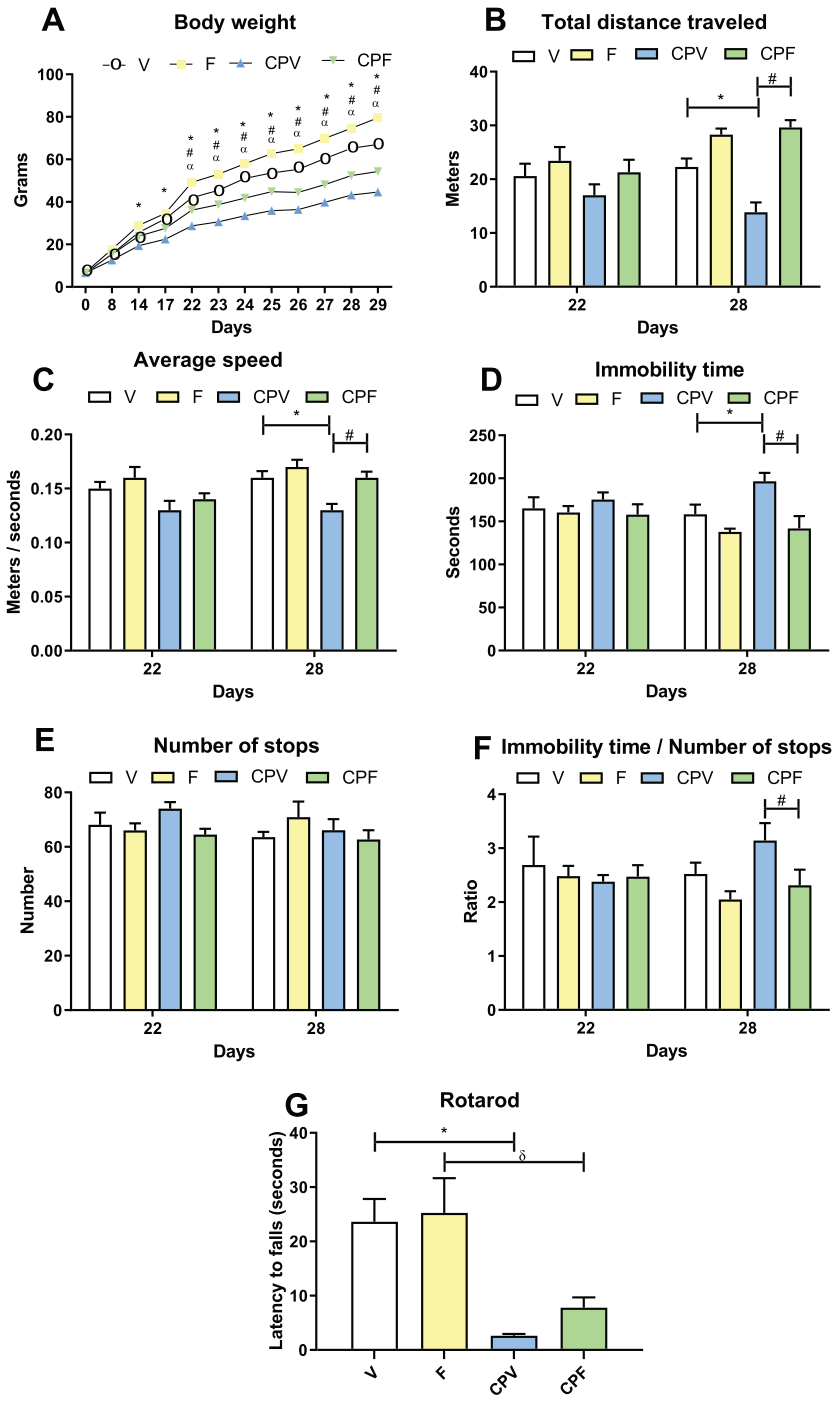

Figure 1 FGF19 treatment increases body weight and preserves locomotor activity, but not motor coordination in cerebral palsy (CP) rats. *(A)* Body weight evolution curves (n = 10–13), *(B)* total distance travelled, *(C)* average speed, *(D)* immobility time, *(E)* number of stops, and *(F)* immobility time/number of stops during locomotor activity tests, before (Day 22) and after (D28) treatment with FGF19 (n = 10 animals per group). *(G)* Motor coordination assessed at Day 29 using the rotarod test (n = 10). V (control + vehicle); F (control + FGF19); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM. $P < 0.05$ for *CPV × V; $^{\#}$CPV × CPF, $^{\delta}$CPF × F, and $^{\alpha}$V × F.

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Results

FGF19 preserves body weight and increases locomotor activity in experimental cerebral palsy

Cerebral palsy rats (CPV and CPF groups) had reduced body weight (*Figure* 1A) and food intake (*Figure* S1) compared with the control non-CP rats (V and F). When treated with recombinant human FGF19, CP animals had higher body weight at the end of the protocol (CPF vs. CPV; *Figure* 1A), but the weight gain during the treatment (D22 to D29) was not significantly different (CPF = 18.2 ± 0.7 vs. CPV = 15.9 ± 1.0 g taken during the treatment period, $P = 0.445$). The body weight gain in the non-CP groups was increased in the presence of FGF19 ($F = 30.3 \pm 1.0$ vs. V = 25.2 ± 1.0 g during the treatment period, $P = 0.008$). There was no significant change in food consumption in response to FGF19 in non-CP and CP animals (*Figure* S1).

At P22, open field experiments revealed no locomotion differences between groups (*Figure* 1B–1F). In contrast, open field records obtained at P28 showed that CPV group had a shorter distance travelled (*Figure* 1B), lower average speed (*Figure* 1C) and longer immobility time (*Figure* 1D) compared with the V group (all with $P < 0.05$). No difference was observed between the four groups in terms of the number of stops (*Figure* 1E). Importantly, rats in the CPF group had and almost complete restoration of their locomotor activity, with parameters globally similar to the control animals (V or

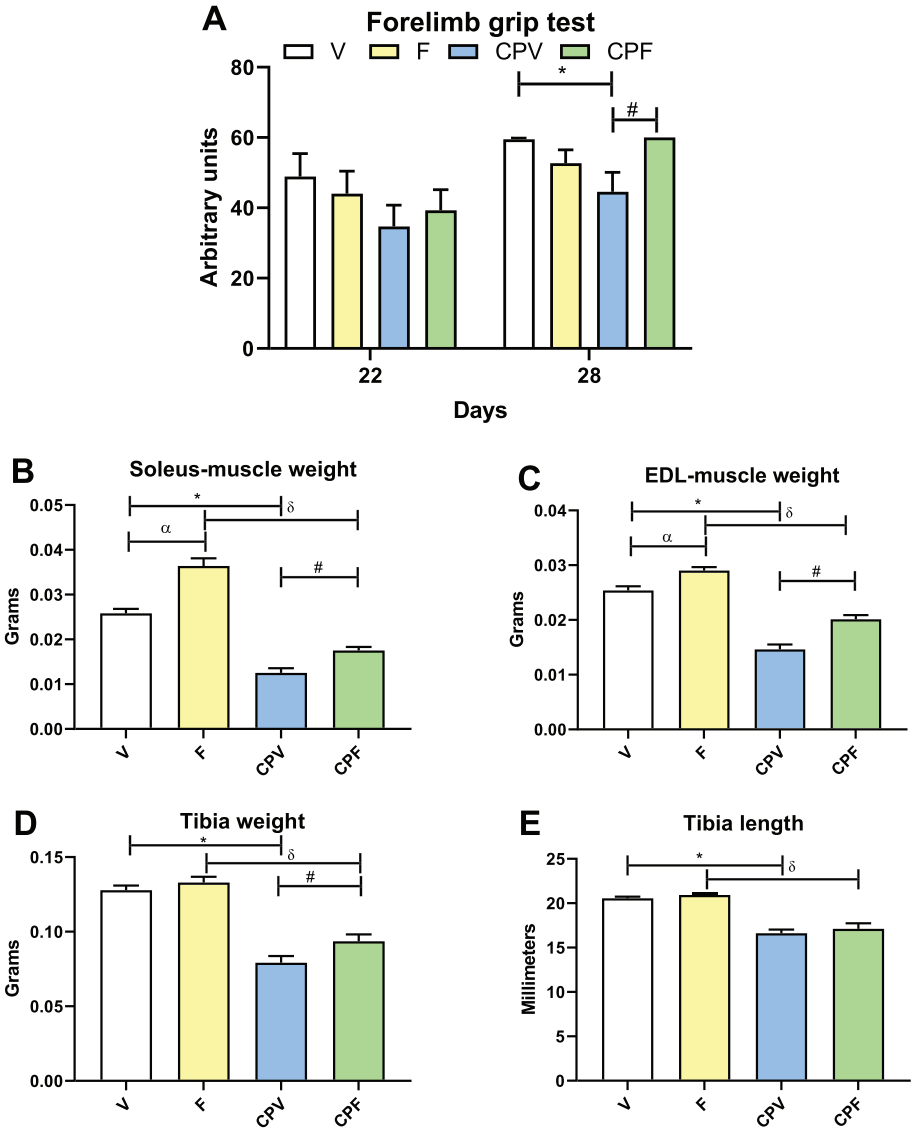

Figure 2 FGF19 treatment increases muscle strength and the weight of skeletal muscles and tibia bone in cerebral palsy (CP) rats. *(A)* Forelimb grip test ($n = 10$), *(B)* soleus weight, *(C)* extensor digitorum longus (EDL) weight, *(D)* tibia weight, *(E)* tibia length ($n = 10$–13). V (control + vehicle); F (control + FGF19); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM. $P < 0.05$ for *CPV × V; $^{\#}$CPV × CPF, $^{\delta}$CPF × F, and $^{\alpha}$V × F.

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F groups). A video recording showing representative locomotor activity of groups V, CPV, and CPF is available as supporting information (*Video* S1).

When motor coordination tests were performed with the rotarod, animals submitted to CP (CPV and CPF) stayed less time on the rod and fell more rapidly compared to non-CP rats (V and F). In CP rats, treatment with FGF19 (CPF) did not significantly improve motor coordination assessed with this test as compared to CPV (*Figure* 1G).

FGF19 increased muscle strength in cerebral palsy

Compared with V group, animals of the CPV group showed a reduction in muscle strength already at P22, which reached statistical significance at P28 (*Figure* 2A). Treatment with FGF19 significantly increased muscle strength at P28 in the CPF group compared with CPV, with a muscle grip strength reaching values similar to those obtained from non-CP animals (*Figure* 2A).

At the end of the experiment (P29), weights of soleus (*Figures* 2B) and EDL (*Figures* 2C) muscles were lower in the CPV group compared with the V group. Treatment with FGF19 significantly increased soleus and EDL muscle weight in both control (F) and CP (CPF) groups (*Figure* 2B and 2C).

Further, we found that CP rats (CPV and CPF) had decreased tibia weight and length as compared to non-CP rats (V and F) (*Figure* 2D and 2E). The administration of FGF19 in CP animals slightly, but significantly, increased tibia weight (*Figure* 2D) without affecting tibia length (*Figure* 2E).

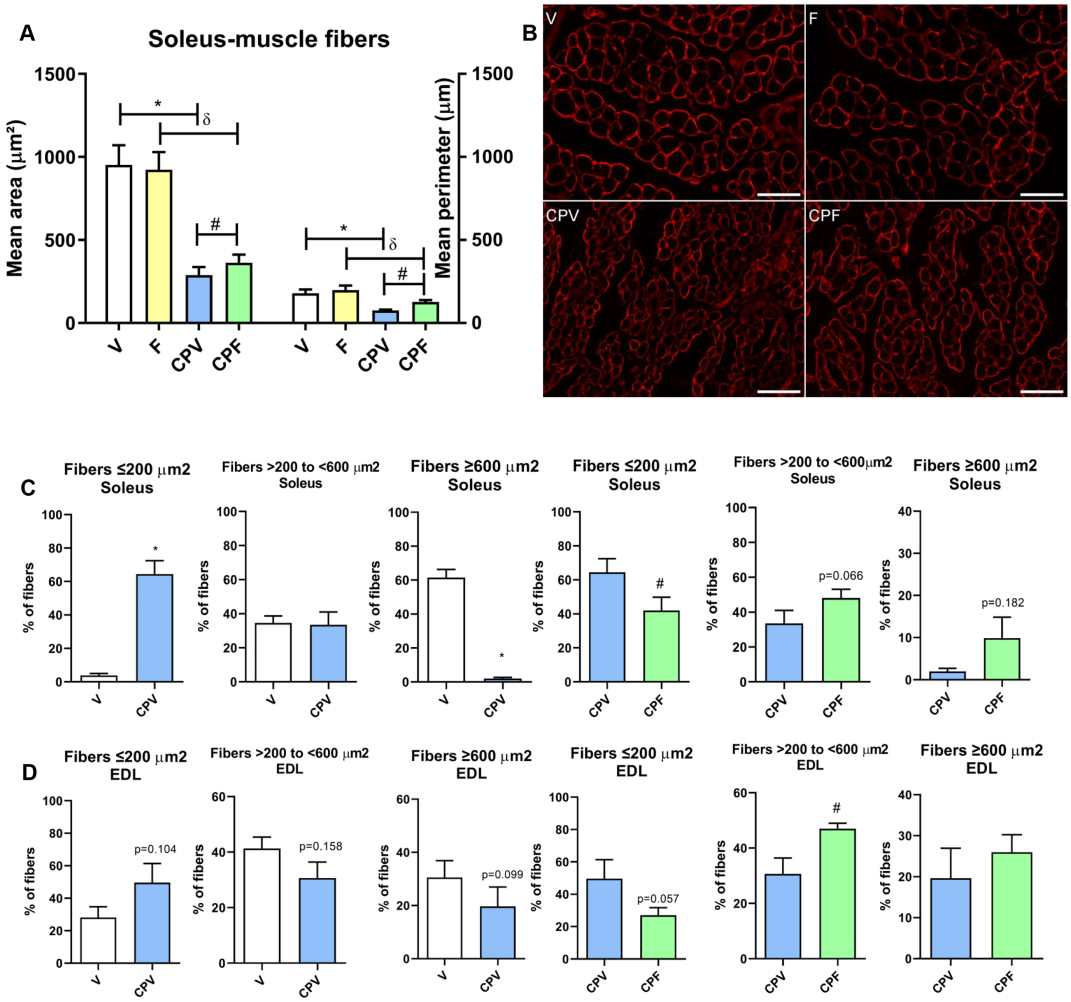

Figure 3 FGF19 treatment affects skeletal muscle fibres size and distribution in cerebral palsy (CP) rats. *(A)* Mean area and perimeter of soleus fibres; *(B)* representative images of laminin-stained soleus muscle (scale bars: 100 µm); *(C)* distribution of cross-sectional soleus muscle fibre area (n = 6–7 animals par group); *(D)* distribution of cross-sectional EDL muscle fibre area (n = 6–7 animals par group). V (control + vehicle); F (control + FGF19); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM. $P < 0.05$ for *CPV × V; $^{\#}$CPV × CPF, $^{\delta}$CPF × F, and $^{\alpha}$V × F.

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FGF19 improves skeletal muscle fibre size in cerebral palsy

In CP animals, soleus muscle fibres were characterized by smaller mean area and perimeter as compared with non-CP animals (*Figure* 3A). In rats submitted to CP, FGF19 treatment increased the mean area and perimeter of the soleus fibres (CPF compared with CPV group, *Figure* 3A and 3B). Distribution of fibre area revealed that rats from the CPV group had a marked increase in very small fibres ($<200\ \mu m^2$) and a dramatic reduction of fibres higher than $600\ \mu m^2$ as compared with V group (*Figures* 3C and S2). Similar tendency was observed in EDL muscle although the difference did not reach statistical significance (*Figures* 3D and S2). When CP animals were treated with FGF19 for 1 week (CPF), the abundance of very small fibres ($<200\ \mu m^2$) decreased in both muscles, and larger fibres reappeared (*Figure* 3C and 3D). There was no difference in the distribution of fibres between V and F (*Figure* S2).

Table 1 Gene expression in skeletal muscles: comparison between human and rat CP and effects of FGF19 treatment

Studied genes	Modifications in human CP (*wrist muscles data*) from[15]	Modifications in rat CP (*soleus or EDL data*) CPV vs. V	Effects of FGF19 in rat CP (*soleus or EDL data*) CPF vs. CPV
Igfbp5	↗	↗	↘
Igf1	↗	↗(tendency)	↘
Dmd	↗	↗	↘
Kcnn3	↗	↗	↘ (tendency)
Gdf8	↗	↗	=
Myh4	↗	↗	=
Neb	↗	=	=
Ucp2	↘	↘	=
Lpl	↘	↘	=
Myod	=	=	=
Myf5	=	↗	↘
Myog	=	↘	=
Musk	Not reported	↗	↘
Nes	Not reported	↗	↘
Pax7	Not reported	↗	=
Tnni1	Not reported	↘	=
Ckmt2	Not reported	↘	=

FGF19 treatment affected the expression of genes in skeletal muscles

The molecular mechanisms occurring in skeletal muscles during CP remain poorly known, but a transcriptomic study has revealed that the expression of a number of genes coding for important proteins and factors involved in skeletal muscle development, myogenesis, and neuromuscular junctions (NJM) are dysregulated in the wrist muscles of children with CP.[15] We therefore measured the expression of some of these genes in the soleus and EDL muscles, and further evaluated whether FGF19 treatment could affect their expression. We found that several genes (9 over 12 tested) displayed similar expression pattern in rat and in human CP (*Table* 1). Indeed, the mRNA levels of *Igfbp5*, *Igf1*, *Dmd*, and *Kcnn3* were increased in soleus or in EDL in CP rats compared with control animals (*Figure* 4). In addition, like in children with CP (Table 1), *Gdf8* (myostatin) and *Myh4* mRNAs levels were increased (*Figure* S3), whereas *Ucp2* and *Lpl*

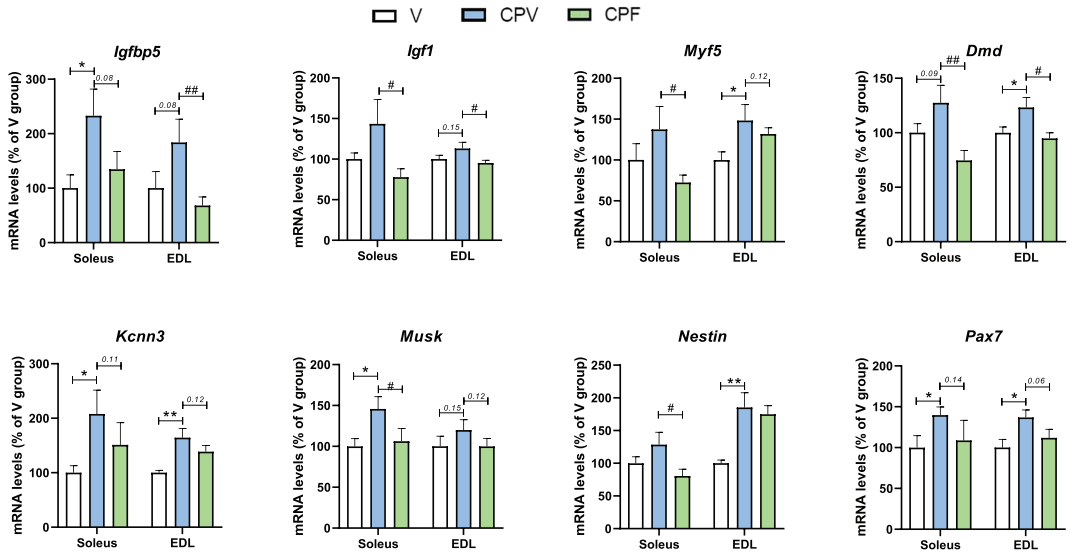

Figure 4 FGF19 treatment regulates the expression of genes altered in human with cerebral palsy (CP) in the soleus and the extensor digitorum longus (EDL) muscle of CP rats. Expression levels of the specific mRNAs were measured by RT-qPCR and normalized to Tbp. The data are presented in % of V group. V (control + vehicle); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM (n = 7–8 different animals per group). $P < 0.05$ for *CPV × V and #CPV × CPF. $P < 0.01$ for **CPV × V and ##CPV × CPF.

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expression levels were decreased in the soleus (these genes were not measured in EDL) (*Figure* S3). In contrast, the change observed in children with CP for nebulin (*Neb*) was not found in rat soleus, and the expression of *Myog* (myogenin) was decreased in soleus of CP rat while it was not affected in patients (*Figures* 4 and S3, Table 1). The other myogenic factors (*Myf5*, *Myod*) were neither modified in children with CP nor in the soleus of CP rats (although *Myf5* was increased in EDL) (*Figure*s 4 and S3, Table 1). From their transcriptomic studies, Smith *et al*. suggested that skeletal muscle were maintained in an immature state during CP, with a possible dysregulation of the NMJ.[15] We found that *Musk* expression, like *Kcnn3*, was increased in the soleus of the CP rat, with a tendency in the EDL (*Figure* 4). We also studied the expression of some additional genes related to muscle differentiation, myogenesis, contraction and metabolism, such as *Pax7*, *Nes* (Nestin), *Tnni1* (Troponin i1), and *Ckmt2* (mitochondrial creatine kinase 2), that were not reported in the human transcriptomic study. Of note, *Nes* and *Pax7* gene expression was increased in both soleus and EDL (*Figure* 4), whereas *Tnni1* and C*kmt2* mRNA levels were decreased in soleus of CP rat compared to control animals (*Figure* S3).

Interestingly, treatment with FGF19 counteracted the CP-associated increased in the expression levels of *Igfbp*, Igf1, *Myf5*, and *Dmd* in the soleus or the EDL muscles (CPF vs. CPV), globally restoring the expression of these 4 genes to levels similar to those observed in the control group (V) (*Figure* 4). Expression of NJM-related genes (*Kcnn3* and *Musk*) and differentiation-associated genes (*Nestin* and *Pax7*) was not significantly affected by FGF19, except for *Musk* and *Nes* mRNA levels that were decreased in soleus only (Figure 4). Other investigated genes in soleus muscle were not modified by FGF19 treatment (*Figure* S3).

The increased mRNA expression of *Pax7* in muscles of CP as compared with V (*Figure* 4) suggested a more immature state of skeletal muscle associated with CP. To confirm these gene expression data, we performed Pax7 immunostaining in soleus muscle samples. As shown in *Figure* 5, muscle of CPV rats showed increased Pax7 staining, confirming the mRNA result. Moreover, treatment with FGF19 did not significantly modify the number of Pax7 labelled cells (*Figure* 5). At the mRNA level, FGF19 tended to reduce *Pax7* gene expression in soleus and EDL, without reaching significance (*Figure* 4). To further investigate whether FGF19 treatment was associated with satellite cell fusion, we evaluated the number of central nuclei in cross-sectional sections of soleus stained with haematoxylin and eosin. Results indicated no significant difference between conditions although there was a tendency (P = 0.12) for a higher number of central nuclei in CP rats (CPV and CPF) as compared to non-CP animals (V and F), with no difference induced by FGF19 treatment (V: 1.1 ± 0.2, F: 1.1 ± 0.4, CPV: 1.5 ± 0.4, and CPF: 2.1 ± 0.4 central nuclei per 100 muscle fibres. Data not shown).

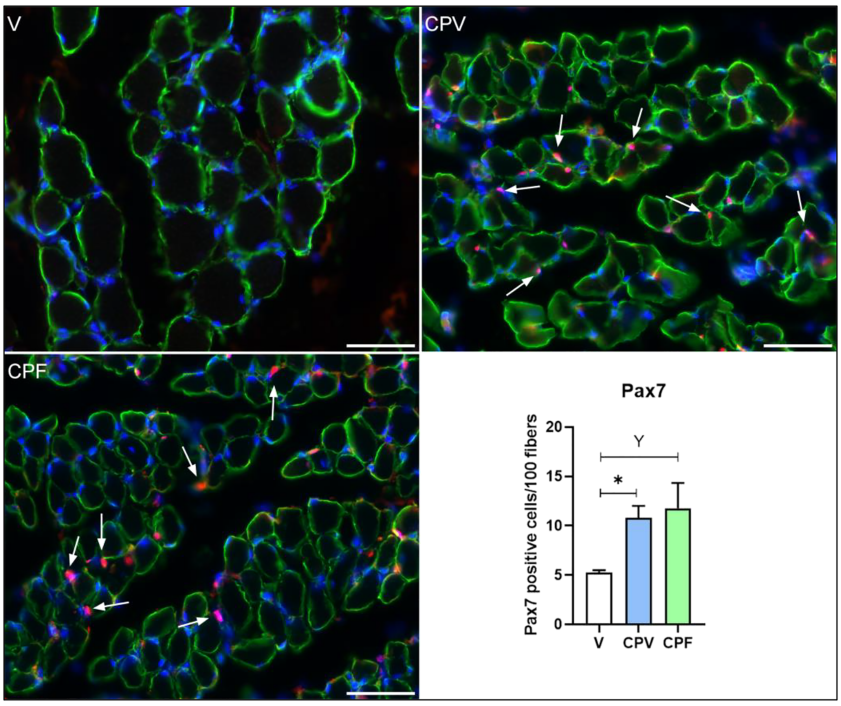

Figure 5 Cerebral palsy (CP) rats have increased number of Pax7 positive cells, which is not affected by FGF19 treatment. Pax7 positive cells were visualized after immunostaining in soleus muscle, counted and normalized by the number of laminin positive fibres (scale bars: 50 μm). V (control + vehicle); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM (n = 7–8 different animals per group). $P < 0.05$ for *CPV × V and ^{Y}CPF × V.

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Discussion

This proof-of-concept preclinical study aimed at evaluating whether a 1 week treatment with human FGF19 could improve motor functions and muscle alterations in an experimental model of CP. Perinatal anoxia associated to restriction of hind paws in rats has been previously reported as a representative model for CP.[7,8,10,11] Evaluated 29 days after birth, the CP animals presented significant defects in locomotion, motor coordination and muscle strength. These damages were associated with lower body weight, smaller area and perimeter of muscle fibres, and reduced bone mass of the tibia. In addition, our analysis of skeletal muscle gene expression revealed similar pattern of alterations than those reported in a genomic profiling study in wrist muscle of patients with CP.[15] Altogether, these data indicated that the experimental CP rat model used in our study closely mimicked the motor disturbances and muscle alterations observed in affected children.

In this study, our strategy was to administer human recombinant FGF19 by daily subcutaneous injections between Days 22 and 28 after birth in the rat model of CP in order to assess the therapeutic potential of FGF19. We found that this treatment improved locomotor activity as well as several musculoskeletal parameters (i.e. area and perimeter of muscle fibres, number of larger fibres, and tibia bone mass) linked to CP. In addition, the expression of several genes that were previously found altered in children with CP[15] was corrected by FGF19 treatment in CP rats. Our data suggest therefore that FGF19 could be a potential novel therapeutic compound against locomotor activity impairments and skeletal muscle weakness associated with CP.

In agreement with preceding reports,[9,10,12] perinatal anoxia and sensorimotor restriction of the posterior limbs affected the development of the animals, as evidenced by a reduction in body weight and weight of muscles and tibia bone. In children with CP, deficiencies in oral feeding and inadequate nutrition are regarded as a major cause of retarded growth and sub-optimal body fat reserves.[17] Here, FGF19 increased muscle and bone weight without affecting food intake. In adult mice, FGF19 treatment is accompanied by a reduction in body weight in obesity models, due to increased energy expenditure.[18,19] However, FGF19 is also known to preserve energy stores by increasing protein and glycogen synthesis in the liver,[20] and we recently discovered that it can also increase skeletal muscle mass in various mouse models.[14] We did not measure glycogen and other parameters in the liver, but we evidenced significant increase in soleus and EDL muscle weight as well as tibia bone. Mechanisms underlying these effects are not known and a potential effect of FGF19 as trophic factor in very young rats, cannot be excluded and remain to be evaluated.

The main defect in the experimental CP group was a marked impairment of locomotor activity, as evidenced both by a reduction in the distance travelled and average speed and by an increase in immobility in the open field test. These observations were consistent with previous studies showing that experimental CP model promotes physical changes interfering with gait performance.[8–10,12] Furthermore, we found a marked decrease in muscle strength using the forelimb suspension test. Reduction in skeletal muscle weight and strength in experimental CP has been previously reported.[7,8,10,12] During the postnatal period, the mechanical forces directed by the muscles adjacent to the bones were also found critical for bone development.[5] We observed that bones were also affected in experimental CP, with a reduction in weight and length of the tibia.

Importantly, the locomotor activity was improved after 1 week of FGF19 treatment. At the end of the treatment, we found that the animals travelled longer distance, had a higher average speed, and had a reduction in the immobility time. In agreement with the recent discovery that skeletal muscle is a direct target of FGF19,[14] we found that treatment with FGF19 increased the weight of soleus and EDL muscles in CP rats, with reduced proportion of very small muscle fibres and increased number of large fibres, and ultimately improved muscle strength. Furthermore, FGF19 treatment increased tibia weight, suggesting that FGF19 may contribute to the interplay between muscles and bones to sustain the development of the musculoskeletal system. Whether FGF19 acts directly on bone or indirectly through its effect on skeletal muscle[14] remains to be determined. Indeed, the literature is scarce regarding the effects of FGF19 on the musculoskeletal system; FGF19 was found expressed in foetal cartilage[21] and a study suggested a potential contribution to growth plate.[22] Whether an action of FGF19 in cartilage could have contributed to the observed increase in tibia weight in young rats remains to be evaluated.

In addition to locomotion defect, experimental CP was associated with a decrease in coordination, which is in agreement with a previous report.[12] Coordination is related to the control of movements, including muscle synergy, in which the neural command activates the co-contraction of specific muscles resulting in the generation of strength and movement in space. Children with CP have deficits in motor planning and execution that do not resolve over time.[23] Similarly, in experimental CP, impaired central brain networks may be responsible for impaired motor coordination.[24] While FGF19 improved locomotion and muscle weight, it did not significantly improve motor coordination as assessed by the rotarod test. This suggested that possible brain damages associated with experimental CP were not affected by treatment with FGF19.

To further shed light on the mechanism of action of FGF19 in skeletal muscle from rats submitted to CP, we performed specific gene expression analyses, using RT-qPCR, in soleus and EDL muscles. Transcriptional profiles

of skeletal muscles from CP patients have been published, identifying several sets of genes with altered expression covering different cellular processes.[15,25] Interestingly, the observed adaptations in gene expression in CP were different from those found in other muscle diseases such as Duchenne muscular dystrophy and muscle atrophy induced by immobilization.[15] Furthermore, comparison of transcriptomic profiles in different muscles (wrist muscles and hamstring muscle) revealed increased expression of genes related to muscle immaturity in human CP.[25] In addition to extracellular matrix and fibre type-related genes, the microarray study in wrist muscles revealed an increase in the anabolic IGF1 (insulin like-growth factor 1) pathway (*Igf1* and *igfbp5* up-regulation), together with an increase in *Gdf8* (myostatin) and *Dmd* (dystrophin) mRNA levels.[15] Of note, one of the most up-regulated genes was *Kcnn3*, encoding the small-conductance calcium-activated potassium channel (SK3) protein.[15] These genes have all been associated with states of muscle atrophy or immaturity in the literature. Indeed, increased *Gdf8* expression has been already associated with skeletal muscle atrophy,[26] and *Kcnn3* gene is expressed in immature muscle cells.[27] Although IGF1 is generally viewed as an anabolic and trophic factor favouring myogenesis, its level is increased in denervated or paralyzed skeletal muscle in rats.[28] We therefore decided to investigate the expression of these genes in the experimental rat CP model. Interestingly, we found that CP is associated with an increase in the expression of *Igfbp5*, *Dmd*, and *Kcnn3*, as well as a tendency for an increase of *Igf1*, in soleus and EDL as compared with non-CP animals. Increased expression of *Gdf8* was also observed in the soleus muscle. Altogether, these data indicated that the molecular characteristics observed in the wrist muscles of patients with CP are conserved in the experimental rat model.

During development, myogenesis is controlled by muscle regulatory factors including myogenin (*Myog*), Myod, and Myf5. Transcriptomic profiling revealed that the expression of these genes was not significantly altered in the muscle of children with CP.[15] In the experimental rat model, we found slightly different results, with no difference in *Myod*, increased expression of *Myf5,* and decreased expression of *Myog*. The myogenic factor Myf5 is among the first signs of myogenesis in mouse embryos and its expression decreases in the late myogenesis stages, when fibres become mature.[29] Myogenin is also involved in the control of the terminal differentiation of myoblasts to myocytes in embryos.[30] These data suggested the presence of more immature muscle cells in the experimental CP. This was also supported by the expression of *Troponin* and of metabolic genes such as *Ucp2*, *Lpl*, and *Ckmt2*, which are generally expressed in mature muscle cells and significantly down-regulated in the soleus muscle of CP rats. Further confirming a retarded development of skeletal muscles in experimental CP, we measured the expression of Pax7, a transcription factor specific of satellite cells and myoblasts, which is classically assessed to estimate the state of differentiation of muscle cells as well as the fusion of myoblasts to form mature fibres.[31,32] Pax7 mRNA levels were increased in both soleus and EDL in rat CP as well as Pax7 immunostaining in soleus supporting therefore a significant increase in the number of satellite cells in skeletal muscles in experimental CP.

Treatment with FGF19 did not modify the number of Pax7 positive cells in the soleus nor the mRNA of *Pax7* gene in the soleus and EDL muscle, indicating therefore that the beneficial effect of FGF19 in muscles was not associated with muscle regeneration or with fusion of satellite cells to form new fibres. This conclusion was also supported by the quantification of the central nuclei in soleus muscle which was not affected by FGF19 treatment, and by the lack of effect on the expression of myogenic factors (*Myog*, MyoD). These results agreed with our previous observations in mouse muscles and in primary culture of human myoblasts showing that FGF19 does not affect myoblast fusion and satellite cells mobilization to sustain its trophic effect on skeletal muscle fibres.[14] In this initial work, we characterized the signalling pathway required by FGF19 to stimulate muscle fibre enlargement. We demonstrated, both *in vitro* and *in vivo*, the involvement of the ERK1/2 mTOR pathway, but we did not identify specific downstream molecular targets in muscle cells.[14] In the present study, focusing on a pathological state with muscle atrophy, we found that the expression levels of several genes that were altered in experimental CP were corrected or restored almost to the control values in response to FGF19 treatment. One of the noticeable observations is that FGF19 significantly decreased *Igf1* and *Igfbp5* expression in the muscles of CP rats, suggesting a possible involvement of an IGF-1 related pathway in the beneficial effects of FGF19. The treatment also decreased the expression of *Dmd* and of *Myf5* in the skeletal muscles, as well of *Nes* and the NMJ-related genes *Kcnn3* and *Musk* in the soleus of CP rats. Increased expression of these different genes have been associated with an immature state of skeletal muscles,[25,26,28,33] and therefore, these data suggested that FGF19 could promote more mature muscles, associated with fibre size enlargement and restoration of muscle strength. However, how FGF19 can interact with these genes and with the IGF1 pathway remains to be investigated, because many overlapping mechanisms could be involved, including central effects increasing locomotor activity in addition to direct action on skeletal muscle.

FGF19 has been suggested to be responsible for growth and invasion of tumours in liver, contributing to hepatocellular carcinoma,[34] thus strongly limiting it therapeutic use in humans.[19] However, a non-mitogenic FGF19 analogue, called Aldafermin (or NGM282) has been developed, and this

engineered form is not able to activate the signalling pathway essential for FGF19-mediated hepatocellular carcinoma, while retaining its ability to regulate metabolism.[35] Safety of Aldafermin in clinical trials has also been evidenced,[36] and despite nothing has been yet reported regarding its possible action on muscle, it might be interesting to envisage its utilization for indications such as CP.

Some limitations of this proof-of-concept study are the duration and window of the treatment, animals being sacrificed at P29, at the end of 1 week of daily treatment with FGF19. We were, therefore, unable to obtain information regarding the medium-term or long-term effects of the treatment, and we cannot ascertain that the observed improvements of locomotion and musculoskeletal system can be maintained overtime. Other periods or durations of FGF19 treatment could also produce different results. Finally, we explored only male animals and additional studies are required to verify whether the beneficial action of FGF19 is observed in both genders.

In summary, this pre-clinical study demonstrates that human recombinant FGF19 therapy could be a novel countermeasure with beneficial effects on locomotion and the musculoskeletal system in a rat model of CP closely mimicking children with CP. Although a number of additional experiments are needed to understand the precise mechanism of action and to demonstrate the long-term benefit of such treatment, our study opens new directions for establishing a possible novel strategy to fight against the locomotor consequences of CP, a highly debilitating neurological disease without efficient treatment.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Food consumption in the experimental cerebral palsy rat model. A) Food consumption (g). Food consumption was estimated by measuring offered diet minus the rejected diet in each day; B) Daily food consumption (g/day): Total food consumption/days assessed; C) Food efficiency coefficient (g/g): Body weight change/total food consumption. V (Control + Vehicle, n = 11); F (Control + FGF19, n = 10); CPV (CP + Vehicle, n = 12); CPF (CP + FGF19, n = 13). Data are expressed as mean ± SEM. *$p < 0.05$ comparing CPV and V; $^{\delta}$ $p < 0.05$ comparing CPF and F.

Figure S2. Frequency distribution of cross-sectional muscle fiber area from soleus and EDL in the different experimental groups.
V (Control + Vehicle, n = 11); CPV (CP + Vehicle, n = 10); CPF (CP + FGF19, n = 10). Data were expressed as mean ± SEM.

Figure S3. RT-qPCR quantification of the expression of a subset of genes in soleus muscle. Levels of the specific mRNAs were measured by RT-qPCR and normalized to Tbp. The data are presented in % of V group. V (Control + Vehicle, n = 7); CPV (CP + Vehicle, n = 8); CPF (CP + FGF19, n = 8). Mean ± SEM. * $p < 0.05$ (CPV vs. V).

Video S1. Representative video showing the locomotor activity in the open field test of a control rat (Vehicle), a rat subjected to cerebral palsy (CP + V) and a CP rat treated with human recombinant FGF19 (CP + FGF19) at 28 days of postnatal life.

Table S1. Sequences of the primers used for RT-qPCR analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

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