Glucose-free/high-protein diet improves hepatomegaly and exercise intolerance in glycogen storage disease type III mice

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Abstract

Glycogen disease type III (GSDIII), a rare incurable autosomal recessive disorder due to glycogen debranching enzyme deficiency, presents with liver, heart and skeletal muscle impairment, hepatomegaly and ketotic hypoglycemia. Muscle weakness usually worsens to fixed myopathy and cardiac involvement may present in about half of the patients during disease. Management relies on careful follow-up of symptoms and diet. No common agreement was reached on sugar restriction and treatment in adulthood.

We administered two dietary regimens differing in their protein and carbohydrate content, high-protein (HPD) and high-protein/glucose-free (GFD), to our mouse model of GSDIII, starting at one month of age. Mice were monitored, either by histological, biochemical and molecular analysis and motor functional tests, until 10 months of age.

GFD ameliorated muscle performance up to 10 months of age, while HPD showed little improvement only in young mice. In GFD mice, a decreased muscle glycogen content and fiber vacuolization was observed, even in aged animals indicating a protective role of proteins against skeletal muscle degeneration, at least in some districts. Hepatomegaly was reduced by about 20%. Moreover, the long-term administration of GFD did not worsen serum parameters even after eight months of high-protein diet. A decreased phosphofructokinase and pyruvate kinase activities and an increased expression of Krebs cycle and gluconeogenesis genes were seen in the liver of GFD fed mice.

Our data show that the concurrent use of proteins and a strictly controlled glucose supply could reduce muscle wasting, and indicate a better metabolic control in mice with a glucose-free/high-protein diet.

1. Introduction

Glycogen storage disease type III (GSDIII; OMIM #232400) is a rare autosomal recessive disease [1,2] caused by deficiency of glycogen debranching enzyme, one of the two enzymes responsible for glycogenolysis. Hepatomegaly, ketotic hypoglycemia, hyperlipidemia, elevated transaminases and failure to thrive are the usual presenting symptoms in the first year of life in GSDIIIa (the most common subtype) [1–3]. Hepatic symptoms usually improve and tend to resolve in adolescence although liver fibrosis and cirrhosis may develop [4,5]. Cardiac involvement, such as left ventricular wall thickness and mass increase, occurs in about half of the patients, but generally is stationary [3,6]. In young adults, myopathy initially presents mostly as exercise intolerance, with involvement of both proximal and distal muscle and elevated CK levels. A fixed myopathy with proximo-distal involvement of variable severity occurs in the following decades, eventually leading to loss of independent walking. Muscle weakness is often described also in young patients revealing that myopathy may occur earlier than usually reported [2].

To date, there is no cure for GSDIII and the current

**Abbreviations:** GSDIII, glycogen disease type III; GDE, glycogen debranching enzyme; HPD, high-protein diet; GFD, glucose-free diet; SD, standard diet; CKs, creatine kinases; BUN, blood urea nitrogen; M, male mice; F, female mice

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recommendations for management rely on follow-up of symptoms and dietary treatment to prevent hypoglycaemia and maintain a constant glycaemia. Particularly, in infancy and childhood fasting hypoglycaemia is less tolerated due to the age-dependent physiology. Euglycaemia is crucial in GSDIII patients but it is not enough to prevent long-term complications such as myopathy and cardiomyopathy [7]. There is not a common agreement about sugar restriction: some authors do not suggest any sugar restriction [1], some others suggest avoiding simple sugars like fructose and lactose in favor of complex carbohydrates [2]. Moreover, carbohydrate overloads should be avoided as it may increase glycogen storage, induce obesity and also insulin resistance [2]. Another emerging challenge is dietary treatment in adulthood.

A number of case reports focused on the improvement of myopathy and/or cardiomyopathy in adults and children that switched their diet in favor of high-protein diet [8–11] or high-fat/ketogenic diet [12–14]. Several benefits come from by using high-protein diet in the treatment of GSDIII: i) the metabolic pathway of gluconeogenesis is not damaged and proteins can be used as source to produce glucose; ii) the increase of dietary protein and the reduction of dietary carbohydrate may decrease glycogen storage in both liver and skeletal muscle; iii) increased protein intake may reduce muscle proteolysis both by increasing the availability of exogenous proteins for energy cell requirements and by enhancing muscle protein synthesis for the maintenance of muscle plasticity [2]. The administration of a high-protein diet in the management of GSDIII has its roots in the Sixties when Fernandes and van de Kamer observed that proteins induce a gradual and prolonged increase in blood glucose [15].

Recently, we developed a knock-out mouse model (GSDIII mouse model) that reproduces the major features of GSDIII like hepato-megaly, progressive glycogen storage in skeletal muscle and liver, and muscle impairment [16]. We administered to GSDIII mice two types of high-protein diet differing in protein and carbohydrate content. Mice fed with glucose-free/low-carbohydrate diet (GFD) showed significant improvement of muscle performance, decreased glycogen accumulation and reduction of hepatomegaly.

2. Material and methods

2.1. Animals and experimental protocols

All studies were approved by the Experimentation Committee (OPBA) of the University of Milan and the Italian Ministry of Health (Authorization number: 1169/2016-PR) and were performed in accordance with Italian guidelines for the use of laboratory animals. Mice were maintained on a 12:12 h light-dark cycle in a temperature- and humidity-controlled environment, and were allowed to free access to standard (SD) or special diets (chow) and water.

The GSDIII mouse model was previously described [16]. Both GSDIII and WT mice were fed with special diets starting from weaning at 1 month of age. High-protein (HPD; 49 kJ% protein, 31 kJ% carbohydrates, 20 kJ% fat) and glucose-free/low-carbohydrate (GFD; 66 kJ% protein, 20 kJ% carbohydrates, 32 kJ% fat) diets were provided by using high-protein diet in the treatment of GSDIII patients. The animal procedures come from by using high-protein diet in the treatment of GSDIII patients. The animal procedures come from by using high-protein diet in the treatment of GSDIII patients.

2.2. Serum analysis and biochemistry

Blood glucose was measured using test strips in a FreeStyle Optium H system (Abbott Diabetes Care). Blood samples were collected from tail vein.

For serum testing and biochemical analysis, mice were anesthetized and blood was sampled by puncture of vena cava in the sub-hepatic tract. The procedure was followed by euthanasia. Serum was sent to Charles River Laboratories for cholesterol, triglycerides, ALT, AST, ALP, creatine kinases (CKs), blood urea nitrogen (BUN) and creatinine determinations.

Glycogen content was determined as previously described [16,17]. The activities of glycolytic enzymes and of enzymes of each respiratory chain complex were measured in tissue homogenates as previously described [18,19]. The activity of each complex was normalized to that of citrate synthase.

2.3. Histological analysis

For light microscopy studies, fresh organs and tissues were processed according to standard methods [20]. Routine stains with and without prior diastase digestion were performed with hematoxylin and eosin and periodic acid–Schiff (PAS).

2.4. Glucose and glycogen metabolism expression profile

We evaluated expression profiling of 84 genes involved in both glucose and glycogen metabolism using the Mouse Glucose Metabolism RT² Profiler™ PCR Array (PAMM-006Z, SABiosciences, QIAGEN). Liver and skeletal muscle (vastus) tissues from 2-month-old mice were analyzed for WT, SD-KO and GFD-KO (n = 4). Equal amounts of total RNA (0.5 μg for liver and 0.8 μg for vastus) were reverse transcribed using the RT² First Strand Kit (QIAGEN). Real-Time qPCR was performed in a 7500 Real Time PCR System (Applied Biosystems). The analysis of expression profiling was performed using the ΔΔCT method using the online free software available at the PCR Array Data Analysis Web portal (www.SABiosciences.com/pcarraydataanalysish.php). Raw data were normalized to the housekeeping genes included in the array. Differentially expressed genes were identified using a 2-tailed t-test and changes in gene expression were presented as fold change increase or decrease (P value < 0.05).

2.5. Statistical analysis

The survival time was calculated using the Kaplan–Meier log rank test. Experimental groups were compared using Student’s t-test and a P value < 0.05 was considered statistically significant. Error bars represent standard deviation.
3. Results

To study the short-term and long-term effects of different protein and carbohydrate loads, GSDIII mice were administered with a high-protein diet (HPD; 45.9% protein) and a second diet with a higher protein content (53.4% protein) and a glucose content < 0.1% of total components (GFD), starting from one month of age. We previously assessed that between 2 and 3 months of age GSDIII mice are susceptible to a decrease in muscle performance, evaluated as their ability to run on a treadmill [16]. Therefore, we established two early time points, at 2 and 3 months of age, to study this critical moment for affected mice, and a later time point, that includes animals between 8 and 12 months of age. Mice fed with HPD or GFD did not show differences in their growth curves respect to mice fed with standard diet (Suppl. Fig. 1A). Also, we did not observe differences between treated or untreated male and female mice.

3.1. Effects of HPD and GFD on liver and heart in GSDIII KO mice

Glycogen content in liver was not significantly reduced neither by HPD nor by GFD respect to untreated mice at any time point (Fig. 1A). A significant glycogen accumulation in hepatocytes was also appraisable by histological analysis at all ages and independently of the type of diet (Fig. 3B, D). The liver glycogen content lowered in WT mice fed with GFD compared to WT mice fed with SD (Suppl. Fig. 4L).

Hepatomegaly, reported as percentage of liver weight on total body weight, was not reduced in HPD-KO mice, indeed, the phenomenon seemed enhanced at least in young ages (Fig. 1B). On the contrary, hepatomegaly was significantly reduced in GFD-treated mice at each time point: the liver was, respectively, 24% ($P < 0.01$), 17% ($P < 0.05$) and 22% ($P < 0.001$) smaller than in untreated mice (Fig. 1B).

Biochemical assessment and PAS analysis of glycogen content in cardiac muscle showed a modestly increased storage in 2-months old HPD-mice (Figs. 1C; 3F, G, K, I). Also, in young HPD-KO mice, heart size appeared to be increased compared to untreated mice at 2 ($P < 0.05$) and 3 ($P < 0.001$) months of age (Fig. 1D). It is noteworthy that the same occurred in HPD-WT mice compared to SD-WT mice ($P < 0.05$; Suppl. Fig. 3G). Conversely, glucose-free diet had no influence on heart size (Fig. 1D), though glycogen content in heart muscle was lower in 2-month old treated mice ($P < 0.01$) than in SD-KO mice (Fig. 1C), as indicated by PAS staining on heart (Fig. 3J-L).

3.2. Effects of HPD and GFD on skeletal muscle in GSDIII KO mice

Biological assessment and PAS analysis of glycogen content in
gastrocnemius and vastus did not show differences between HPD-KO and SD-KO mice at any time point (Figs. 2A, B; 3F, G, K, I). Histological examination of tibialis and diaphragm from these mice evidenced no major differences between the two groups, still showing many vacuoles at any time point (Fig. 4). Conversely, less vacuolization and a better architecture were observed in vastus and gastrocnemius (Fig. 4).

In GFD-KO mice, we observed a reduction of 44% \((P < 0.01)\) of glycogen content in the gastrocnemius at the last time point respect to untreated mice. When vastus was tested, GFD-KO mice showed a statistically significant decrease of glycogen at each time point. HPD did not show differences in glycogen content neither in gastrocnemius nor in vastus except a statically significant increase at 3 months of age. C) Run test. After 10 min of warm-up, mice were run on a treadmill with a 5° incline and a speed of 30 cm/s for a maximum of 5 min (SD-WT \(n = 5\) (3 M, 2F); SD-KO \(n = 10\) (6 M, 4F), except 5-month-old SD-KO \(n = 5\) (3 M, 2F); HPD-KO \(n = 10\) (7 M, 3F); GFD-KO \(n = 9\) (3 M, 6F), except 9 and 10 month-old GFD-KO \(n = 5\) (5F)). HPD-KO mice were able to run for longer time respect to mice fed with SD until 4 months of age. At 5 months of age, both the treated and the untreated groups were able to run just for few seconds. GFD-KO mice showed a substantial and significant improvement of their motor abilities. They were able to run for longer time respect to mice fed with SD and, even better, the muscle performance was maintained for a long period respect to SD-KO mice. At 10 months of age, the muscle performance of the treated group declined but was still higher than muscle performance of untreated 5-month-old mice. Data are shown as mean ± standard deviation. *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\) vs. SD-KO mice.

Histological examination of muscle tissue from GFD-KO mice aged 2, 3 and 9 months, showed a significant reduction in the proportion of vacuolated fibers, in the vastus, gastrocnemius and diaphragm (Fig. 4). Improved morphological features in vastus and gastrocnemius were strikingly evident even in mice aged 9 months. Conversely, the anterior tibialis remained severely compromised, showing large vacuoles that replaced most of the sarcoplasm (Fig. 4).

Muscle performance of treated mice was assessed each month using a motorized treadmill (Fig. 2C). HPD-treated mice were able to run for longer time respect to mice fed with SD until 4 months of age. At 5 months of age, both the treated and the untreated groups were able to run just for few seconds. GFD-KO mice showed a substantial and significant improvement of their motor abilities. They were able to run for longer time respect to mice fed with SD and, even better, the muscle performance was maintained for a long period respect to SD-KO mice. At 10 months of age, the muscle performance of the treated group declined but was still higher than muscle performance of untreated 5-month-old mice.
GFD-KO mice greatly improved their muscle performance running for longer time respect to SD-KO: at 2 months of age, GFD-treated mice run 269 ± 66 s vs. 154 ± 98 s of untreated mice (P < 0.05); at 3 months, GFD-KO mice run 291 ± 23 s vs. 53 ± 32 s of untreated mice (P < 0.001); at 4 months, the outcome was 204 ± 166 s vs. 35 ± 24 s (P < 0.01), and at 5 months, GFD-KO mice run 196 ± 125 s vs. 28 ± 20 s of untreated mice (P < 0.01) (Fig. 2C). Muscle improvement showed by GFD-treated mice allowed them to perform the run test up to 10 months of age, while mice fed with SD or HPD stopped at 5 months. Though GSDIII mice responded very well to GFD, between 8 and 9 months of age their muscle performance lowered and all the mice of the test group leveled off.

3.3. Serum biochemistry in HPD-KO and GFD-KO mice

Both HPD- and SD-KO mice showed transaminase levels higher than WT mice (Suppl. Fig. 2A, B), without significant changes between the treated and the untreated group. The GSDIII mouse model is characterized by lower levels of blood glucose than WT mice in both the fed and the fasted states [16]. HPD-KO mice still had low glycaemia comparable to untreated mice (Suppl. Fig. 2C). BUN and creatinine were investigated to evaluate the impact of the high-protein load on kidneys (Suppl. Fig. 2D, E). BUN was 72% (P < 0.01), 96% (P < 0.01) and 35% (P < 0.05) higher in treated mice at each time point, respectively. An increase of BUN was also observed in WT mice fed with HPD due to the considerable amount of protein intake (Suppl. Fig. 3D, E). Creatinine levels were similar between WT, untreated and HPD-KO mice (WT 0.19 ± 0.03 mg/dL; SD-KO 0.20 ± 0.00 mg/dL; HPD-KO 0.22 ± 0.04 mg/dL; n = 9).

Young GFD-KO mice showed cholesterol levels lower than untreated and WT mice (2-month old GFD-KO mice P < 0.05), whereas triglyceride levels were in the normal range (Suppl. Fig. 4A, B). Transaminase and ALP levels were similar in both treated and untreated group at any time point (Suppl. Figs. 4C–E), as well as glycaemia (Suppl. Fig. 4F). BUN was higher in treated mice at any time point (+55% (P < 0.01), +103% (P < 0.01) and +70% (P < 0.05), respectively) (Suppl. Fig. 4G, H). Creatinine levels were lower in GFD-KO mice respect to both untreated and WT mice (WT 0.19 ± 0.03 mg/dL; SD-KO 0.15 ± 0.05 mg/dL; GFD-KO 0.11 ± 0.03 mg/dL; n = 9). BUN also increased in WT mice fed with GFD, due to the amount of protein intake, but in these mice the increase observed was lower than in GFD-KO and of no statistical significance (Suppl. Figs. 4G, H; 5G, H).

PAS staining of kidney did not show any changes in mice fed with GFD or HPD respect SD-KO mice (Fig. 3M–P). We next evaluated if GFD had effects on the fasting state (Fig. 5). After an overnight fasting, blood samples were collected from GFD-KO mice and SD-KO. Interestingly, when fasted, the latter showed great increase of ALT (SD-KO 114.2 ± 48.8 U/L vs. GFD-KO 22.8 ± 11.0 U/L).
Fig. 4. Histological findings in HPD-KO and GFD-KO mice compared to untreated KO mice. Representative light microscopy images showing muscle tissues from WT, SD-KO, HPD-KO and GFD-KO mice at 2, 3, and 9 months of age. No differences were observed in HPD-KO mice in anterior tibialis and diaphragm, that remained very compromised despite the diet. A slight improvement in the morphological features of vastus and gastrocnemius was noted in HPD-KO mice at all ages examined. In GFD-KO mice both vastus and gastrocnemius showed a marked reduction of vacuoles that was maintained even in older mice. Diaphragm is one of the most compromised muscles in GSDIII mice since young age. In mice treated with GFD, diaphragm showed both a reduction of vacuolization and a better preservation of muscle architecture at any time point. Conversely, the anterior tibialis remained a highly compromised muscle. Bar: 40 μm.
GFD-KO mice 

399.6 ± 278 U/L, L, P

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mice, and a stronger decrease of cholesterol (showed little increase of these values compared to unfasted GFD-KO KO 83.2 ± 12.7 U/L, GFD-KO 54.2 ± 42.5 U/L) (Fig. 5C vs. 

and glucose (increased in fasted SD-KO mice, while it decreased in fasted GFD-KO mice (SD-KO 39.2 ± 2.1 mg/dL vs. GFD-KO 32.0 ± 3.7 mg/dL, P < 0.01), likely due to both the stop of protein intake and the sparing of muscle proteins as energy source which gained importance in a critical condition of lack of nutrients (Fig. 5H). The BUN/creatinine ratio decreased by GFD (Fig. 6). After entering the cell, glucose is addressed to different metabolic pathways according to cell requirements: i) catabolism through glycolysis when energy is needed; ii) conversion in pentose phosphates; iii) storage as glycogen when the cell does not need more energy; iv) or release in the blood flow when glycaemia decreases. Liver and skeletal muscle (vastus) tissues were collected from the same animals of 2 months of age.

3.5. Expression profiling of glucose and glycogen metabolism in untreated and GFD-KO mice

We studied the expression profiling of 84 genes involved in glucose and glycogen metabolism in both liver and skeletal muscle to elucidate the metabolic profiling of GSDIII mice and the potential changes induced by GFD (Fig. 6). Except for G (n = 6, 3 M, 3F), fasted GFD-KO mice: n = 5 (3 M, 2F) for each group; fasted SD-KO mice: n = 4 (2 M, 2F) for each group; ad libitum fed mice: n = 3 (2 M, 1F) for each group. Data are shown as mean ± standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001.

3.4. Survival

Since we previously observed that GSDIII mice have reduced survival compared to WT mice [16], we compared the life span of treated and untreated mice (Suppl. Fig. 1B). Kaplan-Meier survival showed a median survival time of 291.0 days (P < 0.01) for HPD-KO mice and a median survival time of 331.5 days (P = 0.44) for GFD-KO compared to 393.5 days for SD-KO mice.

Fig. 5. Metabolic outcomes of GFD in fasted and unfasted mice. A, B) Cholesterol and triglyceride evaluation showed a decrease of cholesterol content in fasted GFD-KO mice, whereas triglycerides were the same in both groups and in both conditions. C-E) ALT, AST and ALP showed a high increase in fasted SD-KO mice, while fasted GFD-KO mice showed only a slight increase in these values respect to ad libitum fed mice. F) CK levels dramatically increased in fasted SD-KO mice. In fasted GFD-KO mice CK level showed only a slight increase not significant. G) As expected, in fasted mice blood glucose was lower than in fed mice. H) Because of the high protein intake, BUN was higher in ad libitum-fed GFD-KO mice than in SD-KO. In fasted GFD-KO mice urea in the bloodstream decreased respect to ad libitum fed GFD-KO mice, on the contrary in fasted SD-KO mice BUN increased. These data highlight that GFD exerts a protective role against muscle proteolysis during fasting. The BUN/creatinine ratio decreased in fasted GFD-KO mice respect to ad libitum GFD-KO mice due to the stop of protein intake.
Expression profiling of GSDIII mice compared to WT mice

A generalized trend of over expression was noticed in glycolysis, gluconeogenesis, glycogen synthesis and glycogen degradation pathways in the liver of GSDIII mice respect to the WT mice (Fig. 6B). In GSDIII mice the expression of genes coding for the key enzymes of glycolysis, Pfk1 and Pfk2, was respectively unchanged and upregulated. Genes encoding for the TCA cycle did not show any significant difference in their expression compared to WT mice. Conversely, Pdk1, Pdk3 and Pdk4 showed an increased expression (fold changes: Pdk1 1.87, Pdk3 2.51, Pdk4 1.87), as described later. Glycogen metabolism was similar to that of WT mice, except for glycogen metabolism, which was significantly decreased (fold change 0.45, P<0.05).

In skeletal muscle (E) showed gene expression profile in untreated Agl-KO mice compared to WT mice. C, F) Heat maps of liver (C) and skeletal muscle (F) showed gene expression profile in GFD-KO mice compared to WT mice. G) Color code for the different pathways analyzed.

Regulation of glucose metabolism
Glycogen synthesis
Glycogen degradation
Regulation of glycogen metabolism
The enzymes shared by glycolysis and gluconeogenesis are in bold. Data are shown as mean ± standard deviation. Liver: WT and SD-KO mice n = 6 (3 M, 3F); GFD-KO mice n = 4 (2 M, 2F); HPD-KO mice n = 3 (2 M, 1F). Skeletal muscle (gastrocnemius): n = 4 for each group (2 M, 2F). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. WT mice; § p < 0.05, §§ p < 0.01, §§§ p < 0.001 vs. SD-KO mice. n.d.: not determined.

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<td>SD-KO mouse</td>
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<tr>
<td></td>
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<tr>
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The enzymes shared by glycolysis and gluconeogenesis are in bold. Data are shown as mean ± standard deviation. Liver: WT and SD-KO mice n = 6 (3 M, 3F); GFD-KO mice n = 4 (2 M, 2F); HPD-KO mice n = 3 (2 M, 1F). Skeletal muscle (gastrocnemius): n = 4 for each group (2 M, 2F). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. WT mice; § p < 0.05, §§ p < 0.01, §§§ p < 0.001 vs. SD-KO mice. n.d.: not determined.

Table 2
Activity analysis of glycolytic enzymes in liver and skeletal muscle from 2-month old WT, SD-KO and treated KO mice.

The enzymes shared by glycolysis and gluconeogenesis are in bold. Data are shown as mean ± standard deviation. Liver: WT and SD-KO mice n = 6 (3 M, 3F); GFD-KO mice n = 4 (2 M, 2F); HPD-KO mice n = 3 (2 M, 1F). Skeletal muscle (gastrocnemius): n = 4 for each group (2 M, 2F). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. WT mice; § p < 0.05, §§ p < 0.01, §§§ p < 0.001 vs. SD-KO mice. n.d.: not determined.

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Table 2
Activity analysis of glycolytic enzymes in liver and skeletal muscle from 2-month old WT, SD-KO and treated KO mice.

The enzymes shared by glycolysis and gluconeogenesis are in bold. Data are shown as mean ± standard deviation. Liver: WT and SD-KO mice n = 6 (3 M, 3F); GFD-KO mice n = 4 (2 M, 2F); HPD-KO mice n = 3 (2 M, 1F). Skeletal muscle (gastrocnemius): n = 4 for each group (2 M, 2F). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. WT mice; § p < 0.05, §§ p < 0.01, §§§ p < 0.001 vs. SD-KO mice. n.d.: not determined.

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needed (Suppl. Fig. 1B). This could be due to increased load on kidneys or to
of both HPD- and GFD-treated mice was lower than in untreated mice
KO mice skeletal muscle metabolism was sustained by the high-protein
phosphofructokinase and pyruvate kinase, indicated that this tissue needs me-
tabolism because of impaired glycogenolysis. Conversely, in GFD-
fructokinase and pyruvate kinase, indicated that this tissue needs me-
colytic enzymes in GSDIII skeletal muscle, primarily phospho-
reduction of liver glycogen phosphorylase activity in GFD-KO respect to
sation of skeletal muscle
fi
duction of the contraction machinery. In contrast, GFD diet has brought
led us to hypothesize that the protein increase induced by HPD was
(Fig. 2C) and reduced the vacuolization in vastus and gastrocnemius
bohydrate (27.5%) diet, slightly improved the muscle performance
sence of active glycogenolysis. In addition, the long-term administra-
tions was increased in GSDIII mice (Table 2). Hexokinase, phos-
tivities. Glycolysis and gluconeogenesis share seven enzymes, whose
activities were increased in GSDIII mice (Table 2). Hexokinase, phos-
fructokinase and pyruvate kinase control the glucose in
ways (expression, allosteric, hormonal regulation), likely accounting
for the di-
ferences detected between gene expression and enzyme ac-
tivities. Glycolysis and gluconeogenesis share seven enzymes, whose
activities were increased in GSDIII mice (Table 2). Hexokinase, phos-
fructokinase and pyruvate kinase control the glucose in
the liver and skeletal muscle (Fig. 6). In most cases, the upregulation (in
the liver) or the downregulation (in the skeletal muscle) observed did
not reach the standard cut-off used in gene expression analysis (2-fold),
but were seemingly sufficient to show a tendency for gene expression in
each tissue. The major problems of our expression analysis, especially
in skeletal muscle, were the low number of samples and their high
variability which produced low statistical significance. However,
this analysis is an important step to understand the differences between
healthy and diseased tissues, and between treated and untreated
sues.

Glucose and glycogen metabolism are fundamental for cell life and
involve many important pathways that are tightly regulated in different
ways (expression, allosteric, hormonal regulation), likely accounting
for the differences detected between gene expression and enzyme ac-
tivities. Glycolysis and gluconeogenesis share seven enzymes, whose
activities were increased in GSDIII mice (Table 2). Hexokinase, phos-
fructokinase and pyruvate kinase control the glucose in gly-
considering the reduced activity of pyruvate kinase, the
increase of these enzyme activities should be due to the activation of
 gluconeogenesis to provide glucose to the whole organism. This hy-
thesis is also supported by the increased expression of the regulatory
genes of gluconeogenesis in the liver (Pcx, Pck1, Pck2, Fbp1).

The upregulation of gluconeogenesis and the inhibition of glycolysis
in the liver confirm the current working hypothesis. The increased in-
take of proteins from GFD, together with null supply of glucose, acts
inhibiting the glycolytic pathway, activating gluconeogenesis, and de-
creasing the use of glucose in favor of amino acid use. Dietary proteins
are also carried in the TCA cycle to produce energy and substrates
needed by the cell, bypassing the inhibition of glycolysis. The 47%
reduction of liver glycogen phosphorylase activity in GFD-KO respect to
SD-KO mice supports this hypothesis. The increased activation of gly-
colytic enzymes in GSDIII skeletal muscle, primarily phospho-
fructokinase and pyruvate kinase, indicated that this tissue needs me-
tabolic energy because of impaired glycogenolysis. Conversely, in GFD-
KO mice skeletal muscle metabolism was sustained by the high-protein
intake and by the glucose produced by the liver, therefore phospho-
fructokinase activity reduced by 20% compared to untreated mice.

The long-term intake of high-protein/low-carbohydrate diets has
been associated with increased mortality in humans, likely related to an
increased cardiovascular risk [23–25], although another study ruled
out the hypothesis that low-carbohydrate/high protein and fat diets are
associated with coronary heart disease [26]. The median survival time
of both HPD- and GFD-treated mice was lower than in untreated mice
(Suppl. Fig. 1B). This could be due to increased load on kidneys or to
increased cardiovascular risk, as in humans. Remarkably, our study
highlights that the median survival mainly decreased in the group
reated with HPD, the less extreme diet, meaning that the problem does
not seem to be related only with the protein load itself.

Today, a number of the genetic and biochemical differences (i.e.
epigenetic regulation, biosynthetic reactions, and signal transduction)
between humans and rodents are still unexplored; therefore, any
comparison must be carefully handled. On the other hand, studying a
rare disease such as GSDIII is complicated, indeed only dietary treat-
ments aiming to improve cardiac signs have been trialed, and affected
subjects were followed for some months or few years. Our findings
demonstrate that it is possible to limit glycogen deposition and skeletal
muscle degeneration acting on nutrition by drastically limiting carbo-
hydrates and increasing proteins. In these conditions, blood glucose did
not drop down indicating that it is possible to limit glucose adminis-
tration in GSDIII.

Supplementary data to this article can be found online at https://
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Transparency document
The Transparency document associated with this article can be
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Author contributions
S.P., S.L. and G.P.C. conceived the study and designed research;
S.P., S.L., G.U., M.R., R.V., A.B. and F.F. collected the data; S.P.,
S.L., M.R., S.C., M.M., N.B. and G.P.C. analyzed the data; S.P. wrote
the paper. All authors reviewed and approved the paper.

Conflict of interest
The authors have no conflicts of interest to disclose.

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