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

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recommendations for management rely on follow-up of symptoms and dietary treatment to prevent hypoglycemia and maintain a constant glycaemia. Particularly, in infancy and childhood fasting hypoglycemia is less tolerated due to the age-dependent physiology. Euglycemia 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 overload 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 hepatomegaly, 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, 2 kJ% carbohydrates, 32 kJ% fat) diets were from ssniff ® (ssniff Spezialdiäten GmbH, Soest, Germany). For detailed diet compositions see Table 1.

A motorized treadmill (Panlab LE8708; Panlab Harvard Apparatus, Spain) was used to assess muscle performance. Before testing, mice were trained for two consecutive days on a 5° incline for 15 min starting at a speed of 5 cm/s and increasing the velocity of 5 cm/s every 5 min. The treadmill is supplied with an electrified grid that administer a mild shock (0.2 mA) to provide motivation. The exercise test was administered after a 5 min of warm up at a speed of 10 cm/s. The mice were tested at 30 cm/s for 5 min on a 5° incline. The test was interrupted after 5 min or when the mouse could no longer keep pace with the belt.

Table 1
Composition of the diets administered in this study.

Nutrients	Standard diet (SD)	High protein diet (HPD)	Glucose free diet (GFD)
	%		
Crude protein	19.1	45.9	53.4
Crude fat	4.8	8.3	11.4
Crude fiber	3.8	5.0	20.3
Starch	35.4	9.6	0.1
Dextrin	n.a.	6.9	–
Sugar (sucrose, glucose)	4.6	11.0	< 0.1
Others	32.3	13.3	14.7
Total	100	100	100

n.a. not available.

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 biochemistry 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 RT^2 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 RT^2 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 $\Delta\Delta C_T$ method using the online free software available at the PCR Array Data Analysis Web portal (www.SABiosciences.com./pcrarraydataanalysis.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 GDSIII 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

Biochemical assessment and PAS analysis of glycogen content in
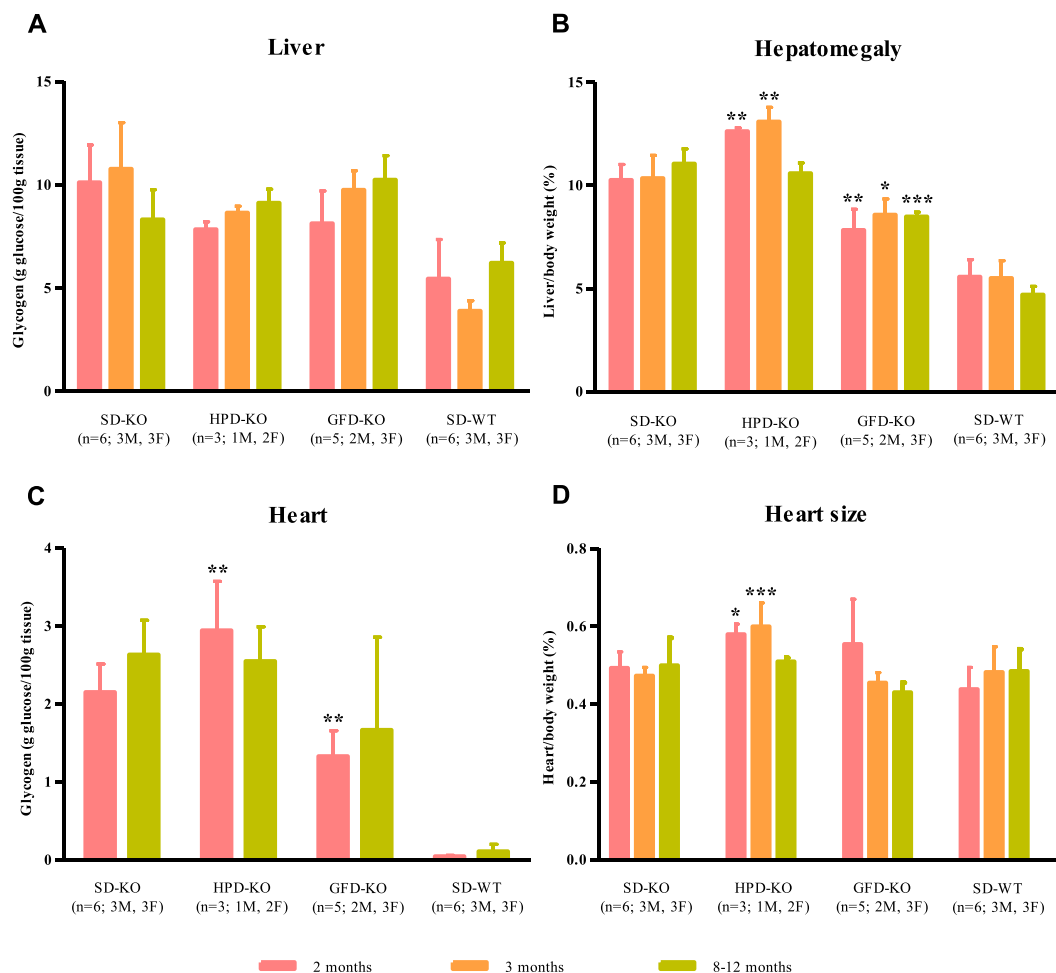
Fig. 1. Short- and long-term effects of HPD and GFD on liver and heart in GSDIII mice. A) Liver glycogen content in HPD- and GFD-KO mice did not show any reduction respect to untreated mice. B) Liver weight expressed as percentage of whole body weight. HPD did not reduce hepatomegaly in treated mice. Instead, hepatomegaly was significantly reduced in mice fed with GFD at any time point showing long-term effects of GFD. C) Glycogen content assessed in heart was not reduced in HPD-treated mice at any time point. GFD-KO mice showed a significant reduction of glycogen storage at 2 months of age respect to untreated mice. D) Heart weight expressed as percentage of whole body weight. In HPD-KO mice of 2 and 3 months of age heart appeared enlarged respect to SD-KO mice, whereas heart size was not different between GFD-KO and SD-KO mice.

Data are shown as mean ± standard deviation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs.* SD-KO mice.
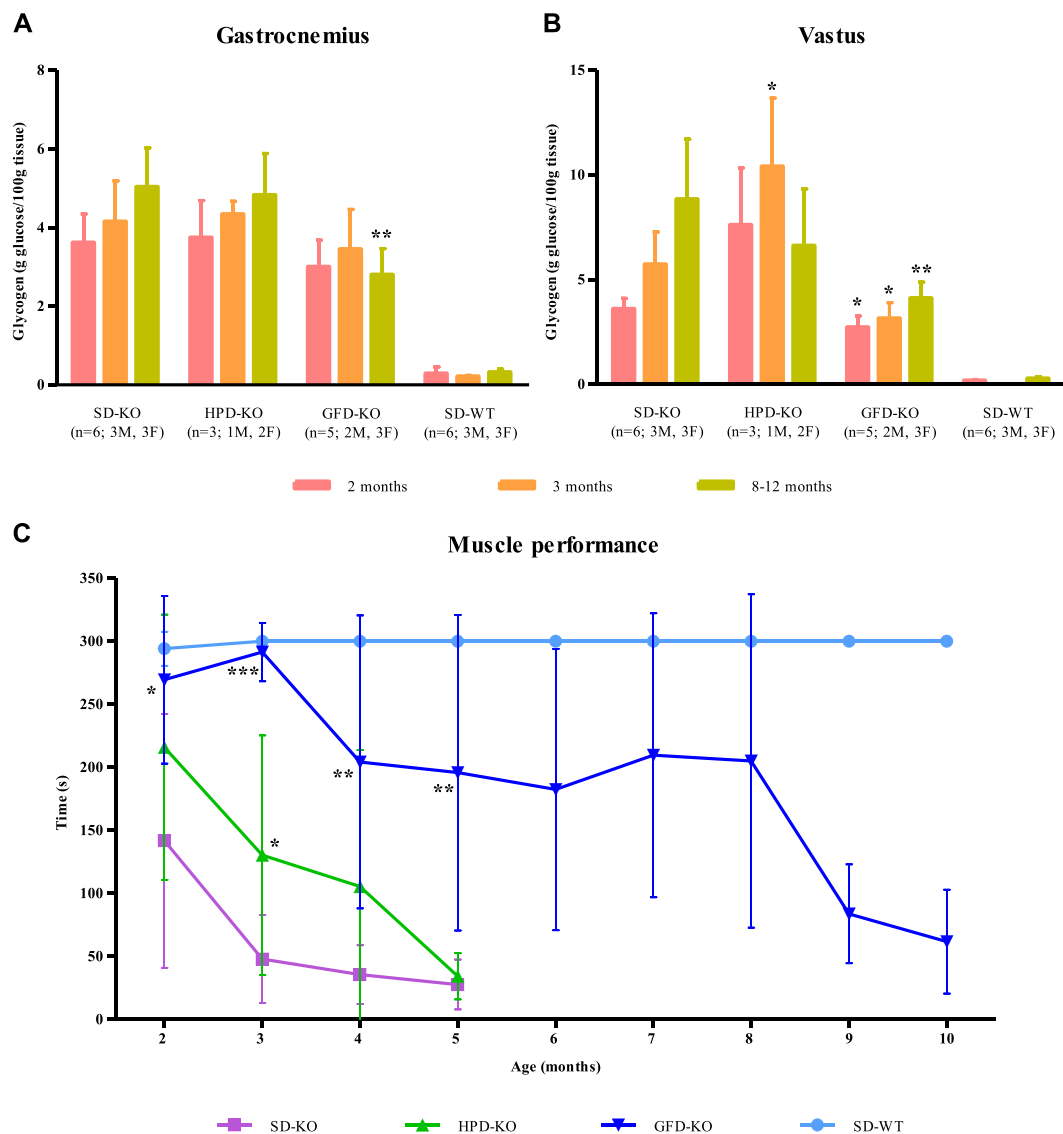
Fig. 2. Short- and long-term effects of HPD and GFD on skeletal muscle in GSDIII mice. A, B) Glycogen content in gastrocnemius showed a significant reduction in GFD-KO mice at the last time point respect to untreated mice. When vastus was tested, GFD-KO mice showed a statistically significant decrease 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.

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 (Fig. 2A). Interestingly, a reduction of glycogen storage of 24% ($P < 0.05$), 45% ($P < 0.05$) and 53% ($P < 0.01$) could be clearly detected in vastus (Fig. 2B). Furthermore, in GFD-WT mice glycogen content in the vastus was almost 50% lower than in SD-WT mice, whereas glycogen content in gastrocnemius did not change (Suppl. Fig. 5K; $P < 0.05$). The whole data set indicated that GFD can limit glycogen accumulation in the

skeletal muscle, at least in some districts. 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 than SD-KO mice at any time point up to 4 months of age, though data were statistically significant only at 2 months of age. Nonetheless at 5 months of age HPD-KO mice had the same disabilities of untreated mice that let them run just for few seconds. Remarkably,

	LIVER	VASTUS	HEART	KIDNEY
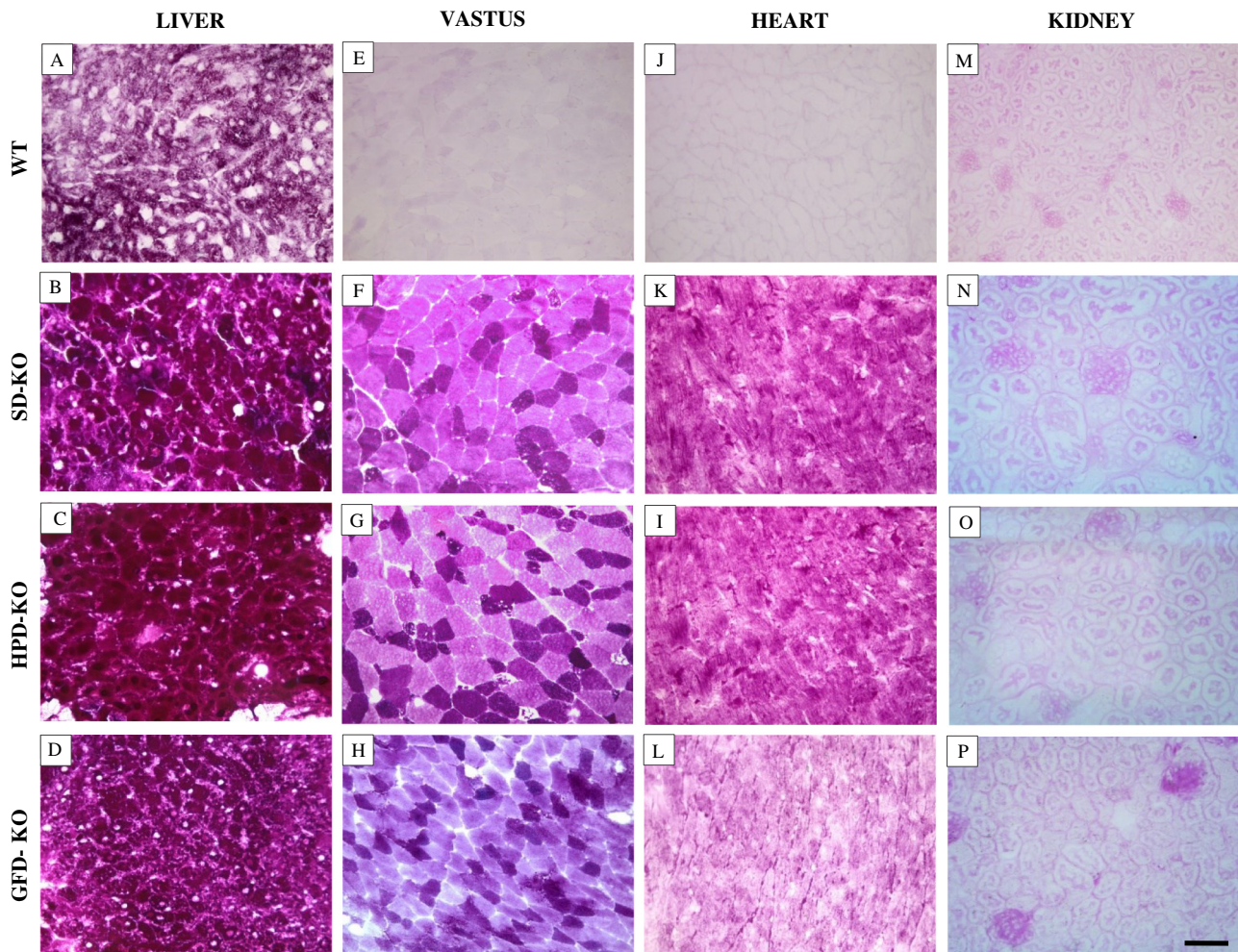
Fig. 3. PAS staining. Liver showed a considerable accumulation of glycogen in all the mice tested, independently of the diet (A-D). PAS staining showed an important reduction in glycogen storage in muscular and cardiac tissues from GFD-KO mice compared to HPD- and SD-KO mice (E-H; J-L). Glycogen content in kidney from SD-KO mice did not show any changes respect to WT mice (M-P). Analyzed tissues were from 3-month old mice. Liver (A-D) and heart (J-L): bar 25 μm. Skeletal muscle (vastus) (E-H) and kidney (M-P): bar 40 μm.

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 ± 20s 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. Fig. 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/
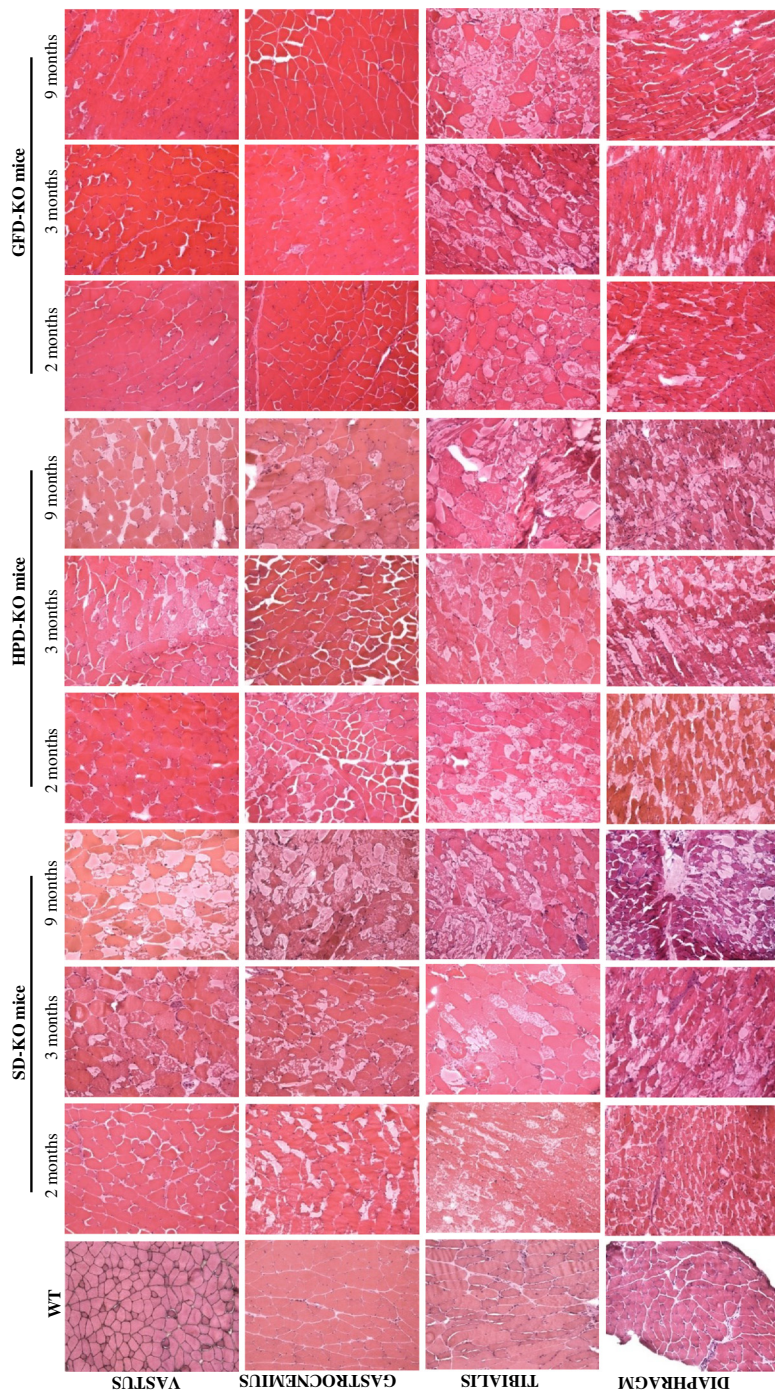
Fig. 4. Histological findings in HPD-KO and GFD-KO mice compared to untreated KO mice. Representative light microscopy images showing muscle tissues from WT, SD-KO, HPD-KO and GFD-KO mice at 2, 3, and 9 months of age. No differences were observed in anterior tibialis and diaphragm, that remained very 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.
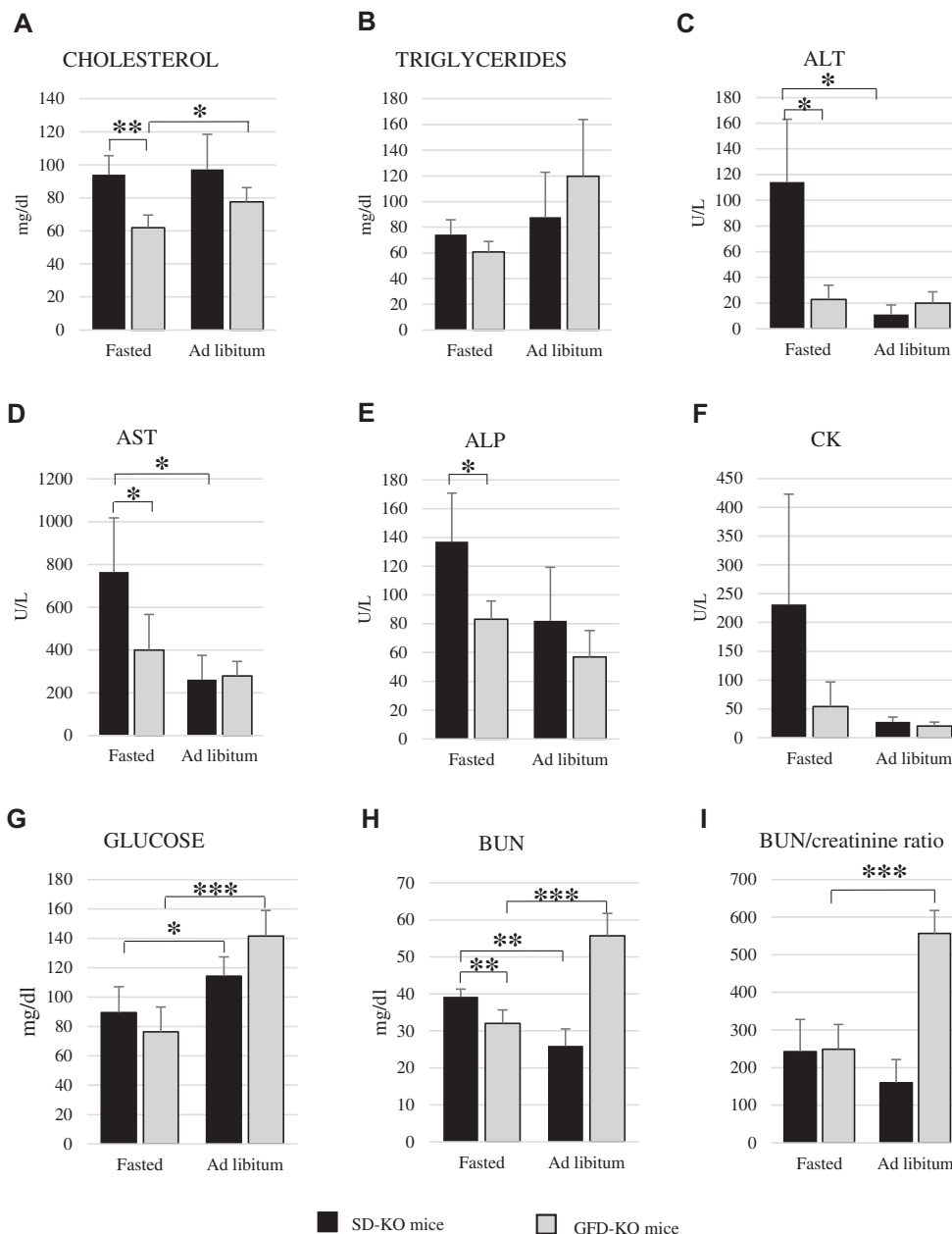
Fig. 5. Metabolic outcomes of GFD in fasted and unfasted mice. A, B) Cholesterol and triglyceride evaluation showed a decrease of cholesterol content in fasted GFD-KO mice, whereas triglycerides were the same in both groups and in both conditions. C-E) ALT, AST and ALP showed a high increase in fasted 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.

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. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

L, $P < 0.05$), AST (SD-KO 765.0 ± 261.7 U/L *vs.* GFD-KO 399.6 ± 278 U/L, $P < 0.05$), ALP (SD-KO 137.0 ± 33.8 U/L *vs.* GFD-KO 83.2 ± 12.7 U/L, $P < 0.05$) and CKs (SD-KO 231.2 ± 191.6 U/L *vs.* GFD-KO 54.2 ± 42.5 U/L) (Fig. 5C–F). GFD-KO mice when fasted showed little increase of these values compared to unfasted GFD-KO mice, and a stronger decrease of cholesterol ($P < 0.05$), triglycerides and glucose ($P < 0.001$) than fasted SD-KO mice (Fig. 5A, B, G). BUN 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 too (Fig. 5I).

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.

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). 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.

A — LIVER

Hk3	Pgk1	Pcx	Acly	Idh1	Suclg1	Mdh2	Ugp2	Gsk3a
Gck	Pgam2	Pck1	Pdha1	Idh2	Suclg2	G6pdx	Gys2	Gsk3b
Galm	Eno1	Pck2	Pdhb	Idh3a	Sdha	H6pd	Gbe1	Phka1
Pgm1	Pklr	Fbp1	Dlat	Idh3b	Sdhb	Rpe	Pygl	Phkb
Gpi1	Pdk1	G6pc	Dld	Idh3g	Sdhc	Rpia	Pgm2	Phkg2
Pfkl	Pdk2	G6pc3	Cs	Ogdh	Sdhd	Tkt	Pgm3	
Aldob	Pdk3	Pdp2	Aco1	Dlst	Fh1	Taldo1	Prps1	
Tpi1	Pdk4	Pdpr	Aco2	Sucla2	Mdh1	Rbks	Prps2	

D — VASTUS

Hk2	Pgam2	Pcx	Acly	Idh1	Suclg1	Mdh2	Gsk3a	Phkb
Gck	Eno3	Pck1	Pdha1	Idh2	Suclg2	G6pdx	Gsk3b	Phkg1
Galm	Pdk1	Pck2	Pdhb	Idh3a	Sdha	H6pd	Phka1	
Pgm1	Pdk2	Fbp2	Dlat	Idh3b	Sdhb	Rpe	Pygm	
Gpi1	Pdk3	G6pc3	Dld	Idh3g	Sdhc	Rpia	Pgm2	
Aldoa	Pdk4	Ugp2	Cs	Ogdh	Sdhd	Tkt	Pgm3	
Tpi1	Pdp2	Gys1	Aco1	Dlst	Fh1	Taldo1	Prps1	
Pgk1	Pdpr	Gbe1	Aco2	Sucla2	Mdh1	Rbks	Prps2	

SD-KO vs. WT mice

B — LIVER

2,25	1,12	1,62	1,00	1,08	1,37	1,24	2,04	1,09
-2,21	1,84	1,78	1,21	1,46	1,28	2,12	1,15	-1,02
1,02	1,62	3,90	1,19	1,35	1,06	-1,24	1,52	1,09
1,51	1,96	1,63	1,09	-1,05	1,12	-1,05	1,31	1,06
1,58	1,87	1,31	-1,00	1,28	1,01	-1,26	1,06	-1,02
-1,14	1,20	1,13	1,21	-1,03	-1,08	1,22	1,65	
1,98	2,51	1,10	-1,16	-1,01	1,32	-1,10	1,04	
-1,05	2,76	1,28	1,24	1,20	1,22	-1,00	-1,03	

E — VASTUS

-1,15	-1,46	-1,29	-1,13	-1,01	-2,33	-1,38	-1,38	-1,28
-1,64	-1,44	-1,19	-1,32	-1,09	-1,15	1,28	-1,09	1,37
-1,40	-1,19	-1,11	-1,45	-1,42	-1,07	-1,57	-1,43	
-1,05	-1,70	1,16	1,49	-1,13	-1,30	-1,12	-1,44	
-1,30	-1,39	-1,32	-1,68	-1,25	-1,22	-1,33	-1,46	
-1,50	1,68	-1,65	-1,40	-1,42	1,05	-1,05	1,04	
-1,18	1,24	-1,75	-1,01	1,27	-1,58	-1,26	-1,52	
-1,16	-1,51	-1,03	-1,67	-1,53	-1,83	-1,62	-1,21	

GFD-KO vs. WT mice

C — LIVER

1,49	1,21	1,33	-1,12	1,48	2,61	2,07	2,91	-1,00
-2,21	2,27	2,53	1,30	2,07	1,30	-1,10	1,12	-1,06
1,02	1,80	2,63	1,21	1,48	1,57	-1,72	-1,05	1,32
1,73	-2,52	2,86	1,07	1,29	1,64	1,00	-1,14	-1,04
1,02	-1,03	-1,10	1,49	1,57	1,29	-2,44	1,28	1,15
-1,16	1,61	-1,05	1,01	1,45	1,48	-1,54	2,47	
1,58	1,78	1,48	1,12	1,41	3,80	-1,48	1,16	
1,19	1,15	1,29	2,02	1,85	1,98	1,11	1,37	

F — VASTUS

-1,40	-1,26	-1,52	1,22	1,21	-1,54	-1,03	-1,30	-1,31
-1,80	-1,17	8,05	-1,21	-1,01	-1,33	1,37	-1,22	-1,26
-1,03	-1,34	1,40	-1,12	-1,43	-1,04	-1,56	-1,15	
1,09	-1,13	1,21	-1,16	-1,21	-1,11	-1,06	-1,39	
-1,15	2,40	-1,39	-1,19	-1,14	-1,20	-1,24	-1,35	
-1,39	1,84	-1,19	-1,22	-1,12	1,01	-1,09	1,13	
1,28	-1,14	-1,29	1,00	-1,05	-1,04	-1,11	-1,33	
-1,20	-1,62	1,11	-1,15	-1,24	-1,19	1,08	1,18	

-2,52 Magnitude of log2(fold change) +3,90

-2,33 Magnitude of log2(fold change) +8,05

G

Color	Pathway
Glycolysis	Regulation of glucose metabolism
Gluconeogenesis	Glycogen synthesis
TCA cycle	Glycogen degradation
Penthose phosphate	Regulation of glycogen metabolism

Fig. 6. Gene expression analysis in the liver and the skeletal muscle of Agl-KO and GFD-KO mice compared to WT mice. A, D) Liver and vastus of 2-month old mice (n = 4, (2 M, 2F)) were examined and the expression profiling of genes involved in glucose and glycogen metabolism was analyzed. B, E) Heat maps of liver (B) and 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.

3.5.1. 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 contrast, *Gck* was significantly downregulated (fold change 0.45, $P < 0.05$). In GSDIII mice the expression of genes coding for the key enzymes of glycolysis, *Pfkl* and *Pklr*, 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 ($P < 0.05$), *Pdk4* 2.76), representing a possible inhibition on pyruvate dehydrogenase complex. The key enzymes of gluconeogenesis, encoded by *Pcx*, *Pck1*, *Pck2* and *Fbp1* genes, were increased, respectively, 1.62 ($P < 0.001$), 1.78, 3.90 ($P < 0.05$) and 1.63 ($P < 0.001$) folds, indicating a possible activation of this pathway. The expression of *G6pc*, encoding for the key enzyme of regulation of glycaemia by promoting the release of glucose in the

blood flow, was similar to that of WT mice as well as for the pentose phosphate pathway. In GSDIII mice the expression of the genes of glycogen metabolism was similar to that of WT mice, except for *Ugp2* that was upregulated 2-fold ($P < 0.05$) and *Pygl* that was slightly increased (fold change 1.31, $P < 0.05$).

In skeletal muscle, genes coding for glycolytic enzymes showed a trend toward a downregulation as well as genes encoding for proteins of TCA cycle, gluconeogenesis and pentose phosphate pathway (Fig. 6E). Moreover, since neither *Pfkm* nor *Pkm*, the muscle isoforms of phosphofructokinase and pyruvate kinase, are included in the array, we chose to further analyze muscle glycolysis by measuring the enzyme activities (Table 2), as described later. Glycogen metabolism was also downregulated, particularly, *Gys1* and *Ugp2* were downregulated almost 2-fold (fold changes: *Gys1* 0.57, $P < 0.01$; *Ugp2* 0.60).

3.5.2. Expression profiling of GFD-KO mice compared to WT mice

The liver from GFD-KO mice showed a generalized overexpression

Table 2

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

Enzyme	Liver				Skeletal muscle (gastrocnemius)		
	WT mice	SD-KO mice	GFD-KO mice	HPD-KO mice	WT mice	SD-KO mice	GFD-KO mice
	Activity μmol/min/mg protein						
Glycogen phosphorylase	0.045 ± 0.007	$0.127 \pm 0.015^{***}$	$0.070 \pm 0.015^{**}$	$0.073 \pm 0.004^{***}$	n.d.	n.d.	n.d.
Phosphoglucomutase	0.11 ± 0.01	$0.16 \pm 0.01^{***}$	$0.12 \pm 0.01^{§§}$	$0.14 \pm 0.01^{**,§}$	0.34 ± 0.04	0.34 ± 0.05	0.38 ± 0.03
Glucose-6-phosphate isomerase	0.88 ± 0.18	$1.97 \pm 0.30^{***}$	$1.08 \pm 0.09^{§§§}$	$1.08 \pm 0.02^{*,§§§}$	2.84 ± 0.20	$3.52 \pm 0.07^{***}$	$3.73 \pm 0.24^{**}$
Phosphofructokinase	0.017 ± 0.002	0.019 ± 0.003	$0.011 \pm 0.002^{*,§§}$	0.019 ± 0.005	0.66 ± 0.19	$1.52 \pm 0.32^{**}$	$1.23 \pm 0.08^{**}$
Aldolase	0.05 ± 0.01	$0.12 \pm 0.01^{***}$	$0.08 \pm 0.01^{**,§§§}$	$0.08 \pm 0.01^{**,§§}$	1.35 ± 0.12	1.54 ± 0.23	1.51 ± 0.14
Glyceraldehyde-3-phosphate dehydrogenase	0.94 ± 0.33	0.87 ± 0.48	1.03 ± 0.22	$1.52 \pm 0.07^{*,§}$	4.29 ± 0.44	$5.08 \pm 0.24^{*}$	5.17 ± 1.42
Phosphoglycerate kinase	1.41 ± 0.12	$2.10 \pm 0.07^{***}$	$1.60 \pm 0.15^{§§}$	$3.20 \pm 0.14^{*}$	2.23 ± 0.14	$2.59 \pm 0.10^{**}$	2.63 ± 0.43
Phosphoglycerate mutase	0.60 ± 0.03	$0.94 \pm 0.07^{***}$	$0.85 \pm 0.06^{***}$	$0.92 \pm 0.02^{***}$	7.18 ± 0.43	7.65 ± 0.55	$8.56 \pm 0.65^{*}$
Enolase	0.44 ± 0.09	$0.79 \pm 0.14^{***}$	$0.58 \pm 0.07^{*,§}$	$0.51 \pm 0.01^{§§}$	2.66 ± 0.15	2.90 ± 0.25	2.97 ± 0.25
Pyruvate kinase	0.14 ± 0.05	$0.09 \pm 0.02^{*}$	$0.04 \pm 0.01^{*,§§}$	$0.03 \pm 0.00^{**,§§}$	3.35 ± 0.07	$3.94 \pm 0.18^{***}$	$4.14 \pm 0.30^{**}$
Lactate dehydrogenase	1.60 ± 0.15	1.77 ± 0.34	1.57 ± 0.15	$1.22 \pm 0.09^{**,§}$	4.15 ± 0.28	4.46 ± 0.21	$4.86 \pm 0.38^{*}$

The enzymes shared by glycolysis and gluconeogenesis are in bold. Data are shown as mean $\pm$ 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.

of both glucose and glycogen metabolism genes respect to WT mice (Fig. 6C). *Gck* was significantly downregulated (fold change 0.45, $P < 0.05$), as in untreated mice. In GFD-KO mice the expression of the key enzymes of glycolysis, *Pfkl* and *Pklr*, was respectively unchanged and 2-fold downregulated. The TCA cycle genes were generally upregulated respect to WT mice, and a number of genes was 2-fold upregulated: *Aco2, Idh2, Suclg1, Fh1, Mdh1* and *Mdh2* (all $P < 0.01$). The *Pdk2, Pdk3* and *Pdk4* showed an increased expression respect to WT mice (fold changes: *Pdk2* 1.61 ($P < 0.01$); *Pdk3* 1.78; *Pdk4* 1.68), but lower than that observed in SD-KO mice. The key enzymes of gluconeogenesis *Pcx, Pck1, Pck2* and *Fbp1* genes were increased 1.33 ($P < 0.05$), 2.53, 2.63 ($P < 0.01$) and 2.86 ($P < 0.001$) fold, respectively, indicating again an activation of the gluconeogenesis pathway that in these mice was sustained by the dietary amino acids. As seen in untreated GSDIII mice, in GFD-KO animals the expression of glycogen metabolism genes did not show differences compared to WT mice.

Skeletal muscle gene expression showed few differences between GFD-KO and WT mice (Fig. 6F). *Hk2* was downregulated ($P < 0.05$) while genes involved in glycolysis were generally downregulated. Again, the activities of glycolysis enzymes were analyzed (Table 2) as described in the next paragraph. Genes involved in TCA cycle, gluconeogenesis and pentose phosphate pathway did not show major differences compared to WT mice. *Gys1* was slightly downregulated ($P < 0.05$).

3.6. Glycogen phosphorylase, glycolysis and respiratory chain activities in liver and skeletal muscle

Liver glycogen phosphorylase activity was 3-fold more active in 2-month old GSDIII mice than in WT mice ($P < 0.001$), while, in GFD-and HPD-KO mice, it was decreased respect to untreated mice, but higher than WT mice (Table 2).

Glycolytic enzyme activities in the liver from SD-KO mice were increased if compared to WT mice with the exception of phosphofructokinase, that was unchanged, and of pyruvate kinase, that was reduced by 35% ($P < 0.05$). Liver pyruvate kinase activity decreased, respectively, by 71.4% ($P < 0.05$) and 78.6% ($P < 0.01$) in GFD-KO and HPD-KO mice compared to WT mice, while phosphofructokinase activity decreased by 35% ($P < 0.05$) only in mice treated with GFD. In both HPD- and GFD-treated mice, the activities of the other glycolytic enzymes were increased when compared to WT, but were reduced when compared to untreated mice (Table 2).

Enzyme activities increased in gastrocnemius from 2-month old SD-

KO mice compared to WT mice (Table 2). In particular, the phosphofructokinase increased by 130% ($P < 0.01$), whereas pyruvate kinase showed a slight increase (SD-KO 3.94 ± 0.18 *vs.* WT 3.35 ± 0.07 μmol/min/mg protein, $P < 0.001$). In GFD-KO mice, phosphofructokinase activity was increased by 86% ($P < 0.01$), whereas pyruvate kinase was 23% more active ($P < 0.01$) compared to WT mice. In SD-KO mice the other glycolytic activities increased compared to WT mice and resulted unchanged or slightly increased in GFD-KO mice.

The activities of respiratory chain enzymes were analyzed in gastrocnemius of 2-month old WT, SD-KO and GFD-KO mice (Suppl. Table 1). We found differences only in the activity of complex II that was slightly reduced in untreated mice compared to WT mice ($P < 0.05$). Complex II activity increased in GFD-KO mice up to reach the activity of WT mice.

4. Discussion

In this study we explored the long-term administration of two kinds of high-protein/low-carbohydrate diet to the GSDIII mouse model. The main finding of the present study is that a glucose-free diet, containing a very limited amount of carbohydrate (< 0.5% of starch and sugar) and a high-protein content (53.4%), improves the exercise tolerance of GSDIII mice (Fig. 2C) and reduces hepatomegaly by about 20% (Fig. 1B).

We demonstrated that cardiac and skeletal muscle glycogen storage may be limited by nutritional management without drop in blood glucose (Figs. 1C; 2A, B; Suppl. Fig. 4 F). GFD-KO mice showed great improvement of exercise tolerance allowing them to run up to 10 months *versus* 5 months of age as for the untreated and HPD-treated mice (Fig. 2C). Histological findings showed a dramatic decrease of vacuolization in the vastus, gastrocnemius and diaphragm of GFD-treated mice up to 9 months of age (Fig. 4). Taken together, these data suggest a protective action of dietary proteins against skeletal muscle degeneration.

A recent study about proteomics of mouse skeletal muscle quantified contractile proteins as 53.6% of total protein mass and proteins of core metabolic pathways as 10% of total protein mass [21]. After two months of GFD administration, the protein content in skeletal muscle in treated mice was significantly augmented (data not shown). It can be speculated that in these mice the protein increase also has led to an increment of contractile proteins.

Furthermore, when fasted, SD-KO mice manifested important differences respect to GFD-KO, suggesting a better metabolic control

during fasting and, again, a protective role against muscle proteolysis in the latter group (Fig. 5). In fact, SD-KO mice presented increased BUN during fasting, likely due to the catabolism of muscle tissue in the absence of active glycogenolysis. In addition, the long-term administration of GFD did not worsen the serum parameters even after eight months of diet (Suppl. Fig. 4).

Administration of HPD, which is a high-protein (45.9%)/low-carbohydrate (27.5%) diet, slightly improved the muscle performance (Fig. 2C) and reduced the vacuolization in vastus and gastrocnemius (Fig. 4). However, muscle function decreased and, at 5 months of age, HPD-treated mice were indistinguishable from the untreated. This has led us to hypothesize that the protein increase induced by HPD was sufficient to provide more energy to sustain exercise but not to protect the liver and muscle from long-term glycogen storage and the disruption of the contraction machinery. In contrast, GFD diet has brought two main benefits: first, a decrease in glycogen buildup and vacuolization of skeletal muscle fibers; second, providing sufficient fuel to sustain exercise through gluconeogenesis. As recently described, GSDIII patients suffer from energy deficiency during exercise caused by impaired glycogenolysis [22].

To define the metabolic changes induced by the GFD we analyzed the expression of genes involved in glycogen and glucose metabolism 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 tissues.

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 activities. Glycolysis and gluconeogenesis share seven enzymes, whose activities were increased in GSDIII mice (Table 2). Hexokinase, phosphofructokinase and pyruvate kinase control the glucose influx into glycolysis. 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 hypothesis 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 intake of proteins from GFD, together with null supply of glucose, acts inhibiting the glycolytic pathway, activating gluconeogenesis, and decreasing 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 glycolytic enzymes in GSDIII skeletal muscle, primarily phosphofructokinase and pyruvate kinase, indicated that this tissue needs metabolic 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 phosphofructokinase 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 treated 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 treatments aiming to improve cardiac signs have been trialled, 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 carbohydrates and increasing proteins. In these conditions, blood glucose did not drop down indicating that it is possible to limit glucose administration in GSDIII.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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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