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Abnormal fatty acid metabolism is a core component of spinal muscular atrophy

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Abstract:	Objective: Spinal muscular atrophy (SMA) is an inherited neuromuscular disorder leading to paralysis and subsequent death in young children. Initially considered a motor neuron disease, extra-neuronal involvement is increasingly recognized. The primary goal of this study was to investigate alterations in lipid metabolism in SMA patients and mouse models of the disease. Methods: We analyzed clinical data collected from a large cohort of pediatric SMA type I-III patients as well as SMA type I liver necropsy data. In parallel, we performed histology, lipid analysis, and transcript profiling in mouse models of SMA.

	Results: We identify an increased susceptibility to developing dyslipidemia in a cohort of 72 SMA patients and liver steatosis in pathological samples. Similarly, fatty acid metabolic abnormalities were present in all SMA mouse models studied. Specifically, Smn2B/- mice displayed elevated hepatic triglycerides and dyslipidemia, resembling non-alcoholic fatty liver disease (NAFLD). Interestingly, this phenotype appeared prior to denervation and denervation alone was insufficient to cause fatty liver. Interpretation: This work highlights metabolic abnormalities as a key feature of SMA, suggesting implementation of nutritional and screening guidelines in patients, as such defects are likely to increase metabolic distress and cardiovascular risk. This study emphasizes the need for a systemic therapeutic approach to ensure maximal benefits for all SMA patients throughout their life.

Title: Abnormal fatty acid metabolism is a core component of spinal muscular atrophy

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Abstract

Objective: Spinal muscular atrophy (SMA) is an inherited neuromuscular disorder leading to paralysis and subsequent death in young children. Initially considered a motor neuron disease, extra-neuronal involvement is increasingly recognized. The primary goal of this study was to investigate alterations in lipid metabolism in SMA patients and mouse models of the disease.

Methods: We analyzed clinical data collected from a large cohort of pediatric SMA type I-III patients as well as SMA type I liver necropsy data. In parallel, we performed histology, lipid analysis, and transcript profiling in mouse models of SMA.

Results: We identify an increased susceptibility to developing dyslipidemia in a cohort of 72 SMA patients and liver steatosis in pathological samples. Similarly, fatty acid metabolic abnormalities were present in all SMA mouse models studied. Specifically, *Smn*$^{2B/-}$ mice displayed elevated hepatic triglycerides and dyslipidemia, resembling non-alcoholic fatty liver disease (NAFLD). Interestingly, this phenotype appeared prior to denervation.

Interpretation: This work highlights metabolic abnormalities as an important feature of SMA, suggesting implementation of nutritional and screening guidelines in patients, as such defects are likely to increase metabolic distress and cardiovascular risk. This study emphasizes the need for a systemic therapeutic approach to ensure maximal benefits for all SMA patients throughout their life.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized primarily by motor neuron death. The incidence of SMA is around 1 in 11,000 live births, and most patients rapidly succumb to their symptoms [1]. More than half of patients with SMA have a severe, infant-onset form of the disease with a median life expectancy of 12 months without supportive treatment. SMA is caused by a mutation or deletion in the ubiquitously expressed *Survival motor neuron 1* (*SMN1*) gene [2], which produces a protein (SMN) involved in a number of key cellular pathways, including RNA metabolism and splicing, amongst others (reviewed in [3]).

SMA has traditionally been considered a motor neuron disease. However, this view has evolved as defects in multiple non-neuronal cell types have been identified [4-18]. It is unclear whether extra-neuronal components contribute to the clinical picture of SMA (reviewed in [15, 19]), but they may become particularly relevant for SMA patients where restoration of SMN protein, largely in the nervous system, has been achieved through therapeutic intervention (e.g. Nusinersen; [20]).

Metabolic defects in SMA have been reported previously. Pancreatic defects were observed in mouse models of SMA and in SMA patients [11, 12, 21]. These alterations appear to be the cause of abnormal glucose homeostasis [12, 21]. Furthermore, defects in amino acid metabolism in SMA have been described [22]. Lipid metabolism and fatty acid oxidation defects have also been reported in early studies of patients with SMA [23, 24], where increased esterified carnitine, and reduced □-oxidation capacity are seen [24]. There are also three reports of microvesicular steatosis in livers of patients with SMA [24-26]. The etiology, importance or generalizability of these findings remain unclear. Most recently, non-neuromuscular phenotypes including metabolic defects were reported

prior to their first clinical signs of neuromuscular degeneration in SMA patients [27]. As such, standard of care statements have highlighted the need for further research in metabolic status in SMA patients to inform future nutritional guidelines [28, 29], but strong comprehensive studies are currently still lacking.

The primary goal here is to provide foundational evidence of defects in lipid metabolism in SMA patients and mouse models of SMA. We find an increased propensity of dyslipidemia in SMA patients as well as hepatic fatty deposition. Strikingly, the human findings are reproduced in the *Smn*$^{2B/-}$ mouse model, which develop non-alcoholic fatty liver disease (NAFLD) prior to denervation. Altogether, this work highlights the critical need for investigation of lipid metabolism and the liver in SMA and how this affects the treatment and care of SMA patients in the future.

Methods

Study design

Identification of fatty livers in *Smn*$^{2B/-}$ mice was serendipitous. Upon review of the literature, this sparked a project with the following objectives: Identify whether findings are translated in SMA patients and identify the extent of the fatty acid defect. All objectives were pursued in a simultaneous manner. The data from SMA patients was obtained retrospectively in Italy. Pre-clinical data made use of multiple mouse models of SMA. Serum analysis and lipid quantification were outsourced, and analyses were performed in a blinded fashion. Sample size calculation were not performed for the human data collection as it was retrospective. N number are described in each figure legend. Statistical approach is as described below and in figure captions. Collaboration between laboratories of Kothary and Parson and colleagues occurred mid-project, given overlapping results that were converging. Hence, the resulting manuscript offers pre-clinical data that have been concordant in two independent laboratories.

Patient data

Infant and young SMA patients were recruited from two clinical referral centers for SMA in Italy (UO Neurologia dello Sviluppo, Fondazione IRCCS Instituto Neurologico Carlo Besta, Milan, Italy and SAPRE-UONPIA, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy). The study protocol was approved by institution ethics review boards (University of Milan #7/16 and Carlo Besta Neurological Institute Foundation #37/2016). All patients with blood work results have genetically confirmed diagnosis of SMA and they were explained benefits and risks of the study, and consented to the study. None of the patients were enrolled in any clinical trials at the time of the blood draw. This study used cut-off values proposed by the National

Cholesterol Education Program (NCEP) [30]. Adult dyslipidemia cut-off values were extracted from the third report of the National Cholesterol Education Program (NCEP) expert panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) and The National Lipid Association recommendations for patient-centered management of dyslipidemia: Part 1 - executive summary.

Liver pathology of human necropsies

Necropsies were obtained at BC Children's Hospital (Protocol #H18-00038). Ten cases of human SMA were retrieved from the pathology files of the British Columbia's Children Hospital and the Children's Hospital of Eastern Ontario. Given the timeframe of the necropsies (between 1977 and 1996), patients were diagnosed based on clinical presentation and histological features apart from #6 and #7, which were done after 1992 and were genetically confirmed. After review, two cases were not retained: one had associated features of olivo-ponto-cerebellar atrophy; the other because of negative familial genetic studies performed 16 years later and because of the presence of non-motor changes at histology, in the spinal cord. The autopsies were performed between 1977 and 1996. Slides from the liver were available in all cases. Seven cases were of SMA type I: four females and three males, aged from less than a month to 3 years. One SMA type II case was a female aged 13 years.

Mouse models

The *Smn*$^{-/-}$*;SMN2* (Jackson Laboratory), *Smn*$^{-/-}$*;SMN2*$^{+/+}$*;SMNΔ7* and *Smn*$^{2B/-}$ (wild type BL/6J background) [31] mouse lines were housed at the University of Ottawa Animal Facility and cared for according to the Canadian Council on Animal Care. Experimentation and breeding were performed

under protocol OHRI-1948 and OHRI-1927. *Smn*$^{+/-}$ mice were crossed to *Smn*$^{2B/2B}$ mice to obtain *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ animals. C57BL/6J wild type mice were bred separately. The Taiwanese *Smn*$^{-/-}$*;SMN2* (FVB/N background, FVB.Cg-Smn1^{tm1Hung}Tg(SMN2)2Hung/J from Jackson Laboratory #005058) and *SOD1*^{G93A} mice (B6.Cg-Tg(SOD1*G93A)1Gur/J from Jackson Laboratory #004435) were housed at the Biomedical Sciences Unit, University of Oxford or within Biological Research Resources at the University of Edinburgh. All experiments using mice in the UK were performed in accordance with the licensing procedures authorized by the UK Home Office (Animal Scientific Procedures Act 1986). All tissue for quantitative biochemical analysis were collected at the same time of the day to limit the effect of the circadian rhythm.

Tissue handling and histological analysis

Gross morphology, tissue processing and staining of animal tissues was as described before [13].

Lipid quantification and plasma analysis in mice

Tissue lipid analysis for quantification and profiles were performed at the Vanderbilt Mouse Metabolic Phenotyping Center. Lipids were extracted and analyzed as described [32, 33]. Cholesterol and unesterified cholesterol quantification protocol was adapted from [34]. Following decapitation of the mice, blood was collected via capillary using Microcuvette CB 300 K2E coated with K2 EDTA (16.444.100). All the blood collected in this study was sampled *ad libitum* (i.e. no fasting period) between 9 and 11 am to limit the effect of the circadian rhythm. Samples were then spun at 2000 g for 5 min at room temperature to extract plasma. Lipoproteins analysis were performed at the National Mouse Metabolic Phenotyping Center (MMPC) at the University of Massachusetts Medical School using a Cobas Clinical Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN,

USA). Blood glucose was obtained using blood glucose and ketone monitoring system FreeStyle Precision Neo and FreeStyle precision glucose strips.

Gene expression studies

RNA from liver and skeletal muscle was extracted using Qiagen RNeasy Mini kit and reverse transcribed using RT^2 first strand kit according to manufacturer's protocol. Qiagen microarray fatty acid metabolism (PAMM-007Z) and fatty liver (PAMM-157Z) were used and were analyzed using RT^2 Profiler PCR Array Data Analysis (http://saweb2.sabiosciences.com/pcr/arrayanalysis.php). Automatic selection from Housekeeping group panel was used as a method of normalization.

Statistics

Data are presented as the mean ± standard error of the mean. A two-sided Student's *t* test was performed using Microsoft Excel or Graphpad Prism 7 to compare the means of data when only two groups were compared (i.e. wild type vs. $Smn^{2B/-}$). One-way ANOVA analysis was used to distinguish differences between more than two groups when multiple comparisons were necessary (i.e. wild type vs. $Smn^{2B/+}$ vs. $Smn^{2B/-}$). The post-test used for the ANOVA was Tukey. Significance was set at $P \leq 0.05$ for *, $P \leq 0.01$ for **, $P \leq 0.001$ for *** and $P \leq 0.0001$ for ****. N number for each experiment is as indicated in the figure legends.

Results

SMA patients are at an increased risk of dyslipidemia and fatty liver

We performed lipid profiling on 72 pediatric SMA patients (14 type I, 52 type II, 6 type III – demographics in Table 1). Briefly, the cohort was fairly evenly split between male (54.17%) and female (45.83%). The median age for the whole cohort was 3.8 years, while for males was 3.7 years old and for females was 4 years old. The median time before their last meal was 5 hours. Note that fasting has minimal effect on lipid levels in comparison to non-fasting [35]. We focused on total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), non-HDL and triglycerides to assess abnormalities in fatty acid metabolism in a minimally invasive manner. Over a third (37.5%) of SMA patients, most commonly type I and II, had at least one positive readout out of the five indicative tests for laboratory-defined dyslipidemia (Table 2), in comparison to less than a quarter (20-24%) of the general population in published data sets [36]. Furthermore, close to 20% and 13% of SMA patients had more than 2 or 3 positive readouts out of the 5 tests of laboratory-defined dyslipidemia, respectively (Table 2). LDL prevalence was doubled in comparison to the general pediatric population [36-38]. Patients with borderline values made up 61% of SMA patients with at least one indicative lipid result, and 40% would have 3 or more (Table 2). Notably, 3/8 (37.5%) of pediatric SMA liver necropsies revealed steatosis, reminiscent of the proportion of SMA patients showing dyslipidemia (37.5% as well) (Table 3). This is in marked contrast to reported prevalence of NALFD in the pediatric population 2-19 years of age, estimated to be between 2% and 13% [39, 40]. If we limit the age range to 2-4 years, which is more in line with our SMA necropsy cohort, it has been reported that liver steatosis incidence is only 0.7% in the normal pediatric population [40].

We next assessed whether any associated glucose mishandling defects may be present, as we had previously identified pancreatic defects [11, 12]. We obtained HbA_{1C} data, a measure of the mean glucose level over the previous 3 months, for 53 of the 72 patients in our cohort. Interestingly, HbA_{1C} trended lower in most SMA patients, with 30 out of 53 having an abnormally low readout (HbA_{1C} < 5%, normal 5%-6.5%). In fact, most SMA patients had an HbA_{1C} around 5% as the calculated median was 4.94% and calculated mean 4.93% in our cohort (Table 2). Overall, a large subset of SMA patients show clinical test results consistent with considerable metabolic abnormalities, especially dyslipidemia and fatty liver.

Abnormal fatty acid metabolism in SMA mouse models

To investigate whether fatty acid metabolism defects identified above may be related to SMN depletion, we assessed livers of the *Smn*$^{2B/-}$ mouse model at pre-symptomatic age (postnatal day 4 (P4)) and at symptomatic age (P17-19). The livers from P17-19 *Smn*$^{2B/-}$ mice were paler and displayed microvesicular steatosis (Fig 1A-B, E-G). The level of triglycerides, the main storage form of fatty acids, was 25-fold higher in livers of P19 *Smn*$^{2B/-}$ mice compared to controls (Fig 1H). Triglyceride chain length alterations, especially of long chain fatty acids, were noted in P19 *Smn*$^{2B/-}$ mice compared to controls (Fig 1I-J). Phospholipid, free fatty acid, diglycerides, cholesterol esters, unesterified cholesterol and total cholesterol (Fig 1K-P) in livers of P19 *Smn*$^{2B/-}$ mice showed alterations, indicating a global misregulation in fatty acid metabolism. By comparison, histology (Fig 1C-D), and lipid levels (Fig 1K-P) were unchanged in P4 *Smn*$^{2B/-}$ livers.

Triglyceride levels were determined in 3 additional mouse models of SMA. Fat accumulation was confirmed in livers from symptomatic P9 *SmnΔ7* mice (*Smn*$^{-/-}$;*SMN2*$^{+/+}$;*SmnΔ7*$^{+/+}$) (Fig 2).

Conversely, more severe mouse models of SMA, such as the "Taiwanese" mice (P9) and the *Smn*$^{-/-}$*;SMN2* mice (P5), showed reduced lipid accumulation in the liver compared to control littermates (Fig 2A-B), likely due to the reduced life span of these animals preventing the opportunity for these pathologies to develop (Fig 2D). A fatty acid pathway-focused PCR array in liver of symptomatic *Smn*$^{2B/-}$ and "Taiwanese" mice, revealed several overlapping alterations, suggesting similar pathogenic etiologies (Fig 3). Significantly, lipid metabolism defects are present across multiple mouse models of SMA. The severity, stage of disease progression and genetic background of the mouse models may influence the extent of lipid accumulation in the liver and the pathological presentation.

To better understand the mechanisms underlying the NAFLD in *Smn*$^{2B/-}$ mice, we next examined the progression of fat accumulation in the liver. Interestingly, the liver became progressively fatty starting at P9 (Fig 4A-E). This raised the possibility that muscle denervation, which may start around this time in *Smn*$^{2B/-}$ mice, could be sufficient to induce liver steatosis. However, *SOD1*^{G93A} mice, a well-established model of amyotrophic lateral sclerosis (ALS) that shows widespread denervation, showed no hepatosteatosis at symptomatic age (20 weeks) (Fig 4F-I). Nevertheless, we cannot discount the possibility that denervation may yet contribute to a lower metabolic demand by skeletal muscle or through alterations of metabolic pathways.

Smn$^{2B/-}$ *mice also display dyslipidemia and abnormal fatty acid metabolism in skeletal muscle*

We next assessed whether *Smn*$^{2B/-}$ mice displayed dyslipidemia and fatty acid metabolism abnormalities in other tissues. Analysis of plasma lipoproteins from P19 *Smn*$^{2B/-}$ mice revealed a significant increase in TC, very low density lipoprotein (VLDL - derived from triglycerides) and

LDL, while HDL levels were reduced (Fig 5A-D). Consequently, the ratios of TC/HDL, a measure of increased cardiovascular risk [41] were significantly elevated (Fig 5E). In addition, glucose always trended lower and its level plummeted after P7 in *Smn*$^{2B/-}$ mice (Fig 5F). The clear dyslipidemic profile and low glucose levels align well with our clinical findings in SMA patients. We then determined if the altered lipoprotein in the plasma translated to an increased delivery of lipids to motor neurons and skeletal muscle, two primary targets in SMA pathogenesis. No changes were observed in the spinal cord and *tibialis anterior* muscle of P19 *Smn*$^{2B/-}$ mice (Fig 5G-H). Given that we had previously identified fatty infiltrates in the skeletal muscle of *Smn*$^{2B/-}$ mice [42], we further investigated whether some alterations in lipid metabolism pathways are present despite no change in triglyceride content. A focused fatty acid PCR array showed that a number of genes were misregulated in muscles of these mice (Fig 5I). These data suggest that fatty acid metabolism is dysregulated in different tissues in SMA mice.

Discussion

Studies from three decades ago had identified potential fatty acid defects with dicarboxylic aciduria, and reduced muscle fatty oxidation, with 3 subsequent reports of hepatic fatty vacuolization in SMA patients [23-26]. Despite these initial findings and the recognition for studies in metabolic defects in SMA by consensus statements in the care of SMA patients [28, 29], minimal research has been done to fill this gap. Indeed, systematic research on fatty acid metabolism in SMA has remained largely unexplored and translational foundational data are lacking. We show that patients with SMA are more prone to develop dyslipidemia as well as liver steatosis than the general pediatric population [36-38, 40, 43], with a large subset showing abnormalities when screened with a common lipid and cholesterol panel and in SMA liver necropsies. In pre-clinical models, fatty acid abnormalities were identified in 4 commonly used SMA mouse models. With a focus on the *Smn*$^{2B/-}$ mice, we observe the development of a NAFLD phenotype that does not appear to be caused by the denervation.

In this study, we provide strong evidence for the susceptibility to dyslipidemia and low glycated hemoglobin levels in a large cohort of SMA patients. To our knowledge, this is the first time that such abnormalities are reported in SMA. Current statistics on dyslipidemia in one laboratory-defined measure in otherwise healthy children is estimated to be roughly 20% [36, 43]. Our studies showed that 37% of SMA patients have dyslipidemia. More strikingly, 14% of patients had more than 3 laboratory-defined measures of dyslipidemia, for which prevalence data is sparse in the normal pediatric population but is suspected to be quite low in the absence of familial dyslipidemia. Pathological examination of SMA liver necropsies showed a similar proportion (37.5%) displaying liver steatosis. This is much higher than the prevalence of liver steatosis in 2-4 year old normal

children (0.7%) [40]. The proportion of fatty liver in our SMA samples was closer to fatty liver prevalence reported in older obese pediatric population, where prevalence can range from 28-77% [39]. Additionally, low HbA_{1C}, observed in the majority of our cohort, has been associated with increased all-cause mortality [44] and liver disease [45] in the general population. Given the significant size of our cohort, we suggest that dyslipidemia is an important feature of SMA. Early metabolic studies on SMA patients had focused on urinary organic acids, muscle β-oxidation enzyme function, and plasma acylcarnitine and free fatty acid profiling [23, 24, 26]. These tests are rarely used, are not widely available, and their interpretation requires a specialist's advice, hence making them poor choices in the screening and identification of SMA patients with potential metabolic abnormalities. Our study provides a widely accessible manner to identify and monitor fatty acid metabolic abnormalities in SMA patients that can be acted upon with current cholesterol-lowering therapy if needed. While necropsies were used for the identification of fatty liver in this study, this can be easily determined through ultrasonography, which consists of a widely used and non-invasive imaging modality [46]. In the upcoming years, screening and preventive treatment of dyslipidemia and fatty liver could be particularly important to limit significant co-morbidities, such as cardiovascular and cerebrovascular disease, in the newly aging demographics of treated SMA patients.

While dyslipidemia and liver steatosis were present in a subset of SMA patients, these phenotypes were present in all *Smn*$^{2B/-}$ mice studied. This is perhaps not surprising as the human population is much more heterogenous than congenic mouse colonies. Nevertheless, the consistency of these findings in human and a pre-clinical model make it clear that SMN depletion pre-disposes the organism to fatty acid defects, dyslipidemia and NAFLD. It is likely that other gene

polymorphisms may be required for (or even protect against) the onset and severity of these phenotypes. Similarly, investigation of hepatic fatty accumulation in four different mouse models of SMA all revealed abnormalities, following an interesting pattern of presentation. It appears that the severity of disease model and related period of survival critically modulates how the alterations in fatty acid metabolism present, reminiscent of our findings in skeletal muscle [5, 42]. We identified that the most severe mouse models (*Smn*$^{-/-}$;*SMN2* and "Taiwanese" mice) show low lipid content in the liver while less severe and slightly longer lived SMA models (*Smn*$^{-/-}$;*SMN2*$^{+/+}$;*SmnΔ7*$^{+/+}$ and *Smn*$^{2B/-}$ mice) display liver steatosis. Even though liver pathology presentation in the "Taiwanese" and *Smn*$^{2B/-}$ mice differs, we identified 9 genes in a fatty liver PCR array with similar patterns of misregulation. Their expression profile as a whole were in line with a compensatory mechanism to limit further triglyceride accumulation. This suggests that some of the molecular etiologies are likely the same, but sufficient time (i.e. longer survival) and perhaps some genetic modifiers may be required to develop the fat accumulation.

A common question in non-neuronal findings in SMA is whether it is a consequence of denervation. In other diseases where denervation is present, such as in spinal cord injury (SCI) [47] and spinal and bulbar muscular atrophy (SBMA) [48], some reports identify an increased risk for dyslipidemia. However, SCI and SBMA patients are generally older adults and dyslipidemia is well-known to be more prominent with age. Thus, generalizing these results to the SMA population is not obvious. In addition, these reports [47, 48] do not account for prevalence of patients with multiple measures of dyslipidemia, such as in our study. A major limitation of our study was the absence of a denervated patient control group. However, previous studies had shown that fatty

acid alterations in SMA patients could not be explained by denervation alone, as denervated control patients did not display these defects [23].

Nevertheless, to overcome this limitation, we turned to pre-clinical mouse models. We found that denervation, such as in the *SOD1*^{G93A} mouse model of ALS, did not lead to development of liver steatosis. Previous literature in ALS described lipid redistribution rather than accumulation in *SOD1*^{G93A} mice, consistent with our findings [49]. It should be noted that this model is also associated with increased lipid clearance in the periphery, which could abrogate fatty accumulation brought on by denervation [50]. Interestingly, we found that the rate of triglyceride accumulation in the liver increases very rapidly with progression of disease in the mouse, which could reflect the overall denervation status of the animal. Indeed, it is possible that denervation plays some role in induction of this phenotype by changing the metabolic demand of the muscle, modulating the molecular metabolism upon denervation, or through other unknown factors.

In simple terms, we believe that NAFLD develops through the imbalance of input (diet, peripheral lipolysis, *de novo* lipogenesis) and output (export of lipids or usage through β-oxidation). We propose that this phenotype in SMA could be caused by potential perturbation in the pancreas-liver axis (as exemplified by the low blood glucose), potential mitochondrial defects, liver-intrinsic defects, or the interplay of the three. Indeed, it appears that hyperglucagonemia, a characteristic found in *Smn*$^{2B/-}$ mice [12] likely in response to low blood sugar, can lead to peripheral lipolysis of white adipose tissue and increase in circulating lipids [51]. Mitochondrial defects have previously been identified in other cell types as well as in human muscle biopsies [52-54] and could likely contribute to reduce β-oxidation in hepatocytes. Additionally, the unknown splicing deficiency in

the hepatocytes could predispose the cells to fat accumulation. Another possibility, in the context of low glucose, includes severe starvation, which also been linked to NAFLD development [55]. Nevertheless, the NAFLD phenotype observed in *Smn*$^{2B/-}$ mice occurs prior to overt motor dysfunction that would thus not prevent the mice from obtaining appropriate nutrition. Moreover, in the case of SMA patients, they are generally followed by nutritionists to ensure appropriate caloric intake. Further molecular studies should attempt to dissect the etiologies and identify nutritional or therapeutic strategies to abrogate this co-morbidity in susceptible patients.

The extent of fatty accumulation identified in *Smn*$^{2B/-}$ mice is likely to result in functional consequences. The liver is the metabolic factory of the body, producing plasmatic proteins, processing toxins as well as medications, and regulating glucose, lipid, and amino acid homeostasis. Going forward, the functionality of the liver in SMA will be a particularly important consideration in formulation of new therapeutic for SMA. Indeed, pro-drugs metabolized through the liver may not gain optimal levels while those cleared/processed by the liver might harbor more significant hepatotoxicity. In fact, historically there has been only cursory examination of liver function in SMA. Embryonic lethality and iron overload are the main features of liver restricted *Smn* conditional knockout in mice [56]. More recently, increased erythropoiesis, megakaryocyte and platelet production, together with mild iron storage abnormalities, were identified in the severe "Taiwanese" mouse model of SMA [10].

Altogether, our clinical studies in SMA patients as well as in preclinical mouse models, provide strong evidence of defects in fatty acid metabolism. The greater predisposition to develop dyslipidemia and fatty liver in SMA patients as well as the identification of NAFLD in *Smn*$^{2B/-}$

mice emphasize that defects in metabolism can lead to added co-morbidities, especially in the new therapeutic era of SMA, where lifespan is extended. Indeed, this work further highlights the importance of establishing currently lacking nutritional guidelines, performing early screening for metabolic defects in treated SMA patients, as well as developing systemic therapeutic strategies that incorporate non-neuronal organs to ensure overall optimal management of SMA.

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Author contribution

MOD, MB, THG, SHP, and RK contributed to the conception and design of the study; MOD, AB, GB, AL, RDA, Alberto B, JWC, HJM, YTH, NLC, AJM, SK, PC, LMM, MB, and SB contributed to the acquisition and analysis of data; CM participated in the enrollment of patients; JM, CD, and KS contributed to the retrieval of pathological specimens and analysis; MOD and RK contributed to the drafting of the text and preparing the figures. GB, HJM, PC, LMM, MB, THG, SB, and SHP read and edited the manuscript.

Potential conflicts of interest

Marc-Olivier Deguise received honoraria and travel accommodations by Biogen for the SMA Summit 2018 held in Montreal, Canada. Rashmi Kothary and the Ottawa Hospital Research Institute have a licensing agreement with Biogen for the *Smn*$^{2B/-}$ mouse model. All other authors have no competing interests to declare.

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Figure Legends

Figure 1. *Smn$^{2B/-}$* mice have fat accumulation in the liver. Gross morphology (0.75X) and histology (H&E - 40X, Oil Red O - 400X) of *Smn$^{2B/-}$* livers showing fatty inclusions at P17-19 (A-B,E-G) but not P4 (C-D). Lipid profiling identified elevation of triglycerides at P19 in *Smn$^{2B/-}$* livers (H), with altered chain length (I-J). Other lipid classes, such as phospholipid, free fatty acids, diglycerides, cholesterol esters, unesterified cholesterol and total cholesterol, were also misregulated in P19 *Smn$^{2B/-}$* livers (K-P). P4 lipid levels were unchanged from control (H, K-P). Scale bar: (A-D) 50 µm, (E-F) 10 µm. (N value for each experiment is as follows: N = 5-6 for A-D, 3-5 for E-G, 4 for H-P, unpaired two-sided student's t-test, $P \leq 0.05$ for *, $P \leq 0.01$ for **, $P \leq 0.001$ for *** and $P \leq 0.0001$ for ****).

Figure 2. Hepatic triglyceride misregulation is a common feature in different SMA models at symptomatic. Quantification of hepatic triglycerides showed a 5 fold reduction in P5 *Smn$^{-/-}$;SMN2* mice (A), a 2 fold reduction in P9 Taiwanese mice (B), and a 3 fold increase in P10 *SmnΔ7* mice (C) in comparison to control littermates. Analysis of hepatic triglyceride levels for each SMA mouse model in A-C involved a comparison to their own control (N value for each experiment is as follows: N = 4-6 for A-B, 9-10 for C, 4-9 for D, unpaired two-sided student's t-test, $P \leq 0.05$ for *, $P \leq 0.01$ for **).

Figure 3. Commonalities identified in expression of fatty acid metabolism genes between *Taiwanese* and *Smn$^{2B/-}$* mice. Volcano plot presentation of all changes (1.5X, $p < 0.05$) in a focused fatty acid metabolism PCR array in *Smn$^{2B/-}$* mice (A) and *Taiwanese* mice (C) identify general downregulation. Changes more than two-fold are represented for *Smn$^{2B/-}$* (B) and

Taiwanese (D). Analysis of commonalities between *Smn*$^{2B/-}$ and *Taiwanese* are represented by Venn diagrams, which identify 9 genes with similar changes (E), listed in (F). (N=4, for *Smn*$^{2B/-}$ mice, and N=3 for *Taiwanese* mice).

Figure 4. Fat accumulation is first observed between P9 and P11 in *Smn*$^{2B/-}$ mice and denervation is not sufficient to trigger hepatic steatosis. (A,B) Triglycerides and cholesterol esters quantification in livers from *Smn*$^{2B/-}$ mice at different ages. (C-E) Oil Red O staining (400X) additionally showed increased fat at P9. (F-H) H&E staining (40X) of livers of 20-week-old *SOD1*^{G93A} mutant mice, a model of ALS, did not show hepatic fat accumulation in comparison to livers from *Smn*$^{2B/-}$ mice, even though denervation is well-established at this time point. (I) Triglycerides and cholesteryl esters quantification showed no difference between mutant *SOD1*^{G93A} and WT controls. Scale bar represents 50 µm in C and D (10 µm in the inset), and in F-H. (N value for each experiment is as follows: N = 4-6 for A and B, 3 for C-E, and 3-5 for I, unpaired two-sided student's t-test, $P \leq 0.05$ for *, $P \leq 0.01$ for **, $P \leq 0.001$ for ***).

Figure 5. *Smn*$^{2B/-}$ mice display dyslipidemia and abnormal fatty acid metabolism in skeletal muscle, but not in spinal cord. (A-D) Significant up-regulation of total cholesterol, VLDL and LDL in the plasma of *Smn*$^{2B/-}$ animals, while HDL levels were significantly lower. (E) Parameters for cardiovascular risks such as TC/HDL were significantly increased for *Smn*$^{2B/-}$ mice. (F) Glucose trend lower early and plummet later in life in *Smn*$^{2B/-}$ mice. (G,H) Every lipid class in the P19 *Smn*$^{2B/-}$ skeletal muscle or spinal cord were at similar levels to WT. (I) Many genes involved in fatty acid metabolism were altered in P19 *Smn*$^{2B/-}$ skeletal muscle in a focused fatty acid PCR array. (N value for each experiment is as follows: N = 10 for A-F, 5 for G, 4 for H-I, unpaired

two-sided student's t-test, $P \leq 0.05$ for *, $P \leq 0.01$ for **, $P \leq 0.001$ for *** and $P \leq 0.0001$ for ****).

Table 1. SMA pediatric patient cohort demographics

Pediatric cohort	Number	Percentage	Median age (years)	Time before last meal (hours)
Total	72	100	3.8	5
Male	39	54.17	3.7	5
Female	33	45.83	4	5
Type I	14	19.44	3.1	5
Male	5	6.94	2	5
Female	9	12.5	3.2	5
Type II	52	72.22	3.8	5
Male	31	43.05	3.7	5
Female	21	29.17	4.2	5
Type III	6	8.33	6.4	5
Male	3	4.17	6.2	5
Female	3	4.17	6.6	5

Table 2. SMA patients are more susceptible to dyslipidemia than the normal population

	Criteria	*All SMA patients*	*Type I*	*Type II*	*Type III*	*Normal population**
Abnormal	*TC > 200 mg/dl*	10/72 (13.89%)	1/14 (7.14%)	9/52 (17.31%)	0/6 (0%)	7.7-10.7% [36-38]
	LDL > 130 mg/dl	9/72 (12.5%)	1/14 (7.14%)	7/52 (13.46%)	1/6 (16.67%)	3.2-7.2% [36-38]
	HDL < 40 mg/dl	12/72 (16.67%)	1/14 (7.14%)	10/52 (19.23%)	1/6 (16.67%)	4.1-19.3% [36, 38, 43, 57]
	TG > 100 mg/dl[♂]	15/72 (20.83%)	5/14 (35.71%)	7/52 (13.46%)	3/6 (50%)	13.2-22.1% [36, 38, 57]
	Non HDL-cholesterol > 145 mg/dl	10/72 (13.89%)	1/14 (7.14%)	8/52 (15.38%)	1/6 (16.67%)	8.4% [43]
	1/5 abnormal dyslipidemia reading	27/72 (37.5%)	6/14 (42.85%)	18/52 (34.62%)	3/6 (50%)	20.2-22.9% [36, 43]
	2/5 < abnormal dyslipidemia reading	14/72 (19.44%)	2/14 (14.29%)	11/52 (21.15%)	1/6 (16.67%)	5.37%[&] [36]
	3/5 < abnormal dyslipidemia reading	10/72 (13.89%)	1/14 (7.14%)	8/52 (15.38%)	1/6 (16.67%)	-
	HbA1C < 5	30/53 (56.60%)	5/8 (62.5%)	23/41 (56.09%)	2/4 (50%)	
Borderline	*TC > 170 mg/dl*	30/72 (41.67%)	5/14 (35.71%)	23/52 (44.23%)	2/6 (33.33%)	-
	LDL > 110mg/dl	21/72 (29.17%)	2/14 (14.29%)	18/52 (34.62%)	1/6 (16.67%)	-
	HDL < 45 mg/dl	20/72 (27.78%)	5/14 (35.71%)	13/52 (25%)	2/6 (33.33%)	-
	TG > 75 mg/dl[#]	23/72 (31.94%)	7/14 (50%)	13/52 (25%)	3/6 (50%)	-
	Non HDL-cholesterol > 120 mg/dl	32/72 (44.44%)	6/14 (42.86%)	23/52 (44.23%)	3/6 (50%)	-
	1/5 < borderline dyslipidemia reading	44/72 (61.1%)	11/14 (78.57%)	30/52 (57.69%)	3/6 (50%)	-
	2/5 < borderline dyslipidemia reading	35/72 (48.61%)	6/14 (42.86%)	26/52 (50%)	3/6 (50%)	-
	3/5 < borderline dyslipidemia reading	29/72 (40.28%)	6/14 (42.86%)	20/52 (38.46%)	3/6 (50%)	-

* Note that these values were taken from multiple studies and criteria may have varied and not be identical to the present study.

[&]Calculated from results in the particular study – see reference.

[♂]High is defined as >100 for 0-9 years and >130 for 10-19 years of age

[#] Borderline is defined as >75 for 0-9 years and >90 for 10-19 years of age

Table 3. Presence of steatosis in SMA liver necropsies

Case	Sex	Age	Type	Cause Death	Steatosis*
1	F	14 mo	I	Bronchopneumonia	-/**
2	F	7 mo	I	Bronchopneumonia	-
3	M	8 mo	I	Aspiration pneumonia	+++
4	M	< 1 mo	I	Aspiration pneumonia	-
5	F	9 mo	I	Respiratory insufficiency	+/++
6	F	14 mo	I	Bronchopneumonia	-
7	M	12 mo	I	Respiratory insufficiency + HIE + Chronic pneumonitis	+/++
8	F	13 y	II	Undetermined	-

HIE: Hypoxic ischemic encephalopathy

*: -: no steatosis; +: mild panlobular; ++ and +++: moderate and severe, panlobular. In all cases with steatosis (cases 3, 5 and 7), it was of the microvesicular type, predominantly and the periportal regions were more involved than the mid or central regions of the hepatic lobules.

**: Presence of moderate increase of glycogen

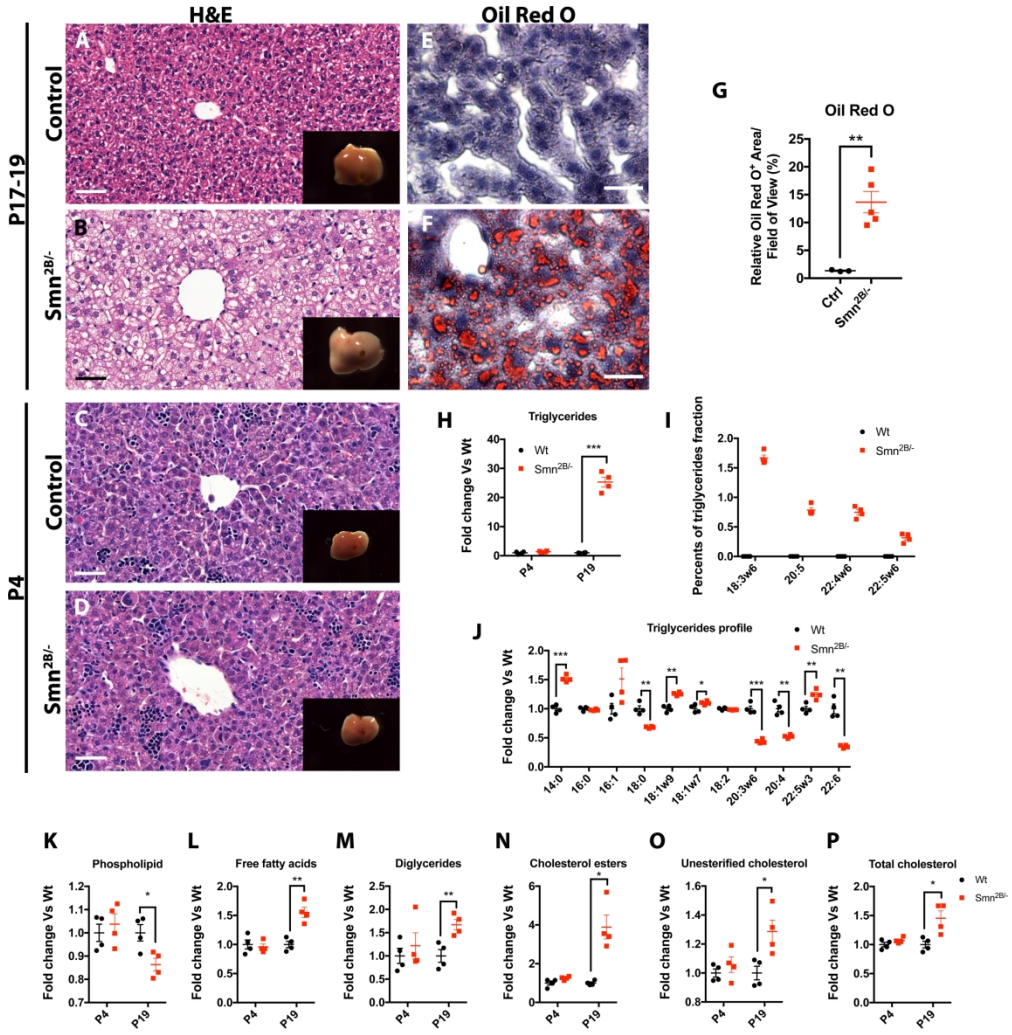


Figure 1. Smn2B/- mice have fat accumulation in the liver.

214x219mm (300 x 300 DPI)

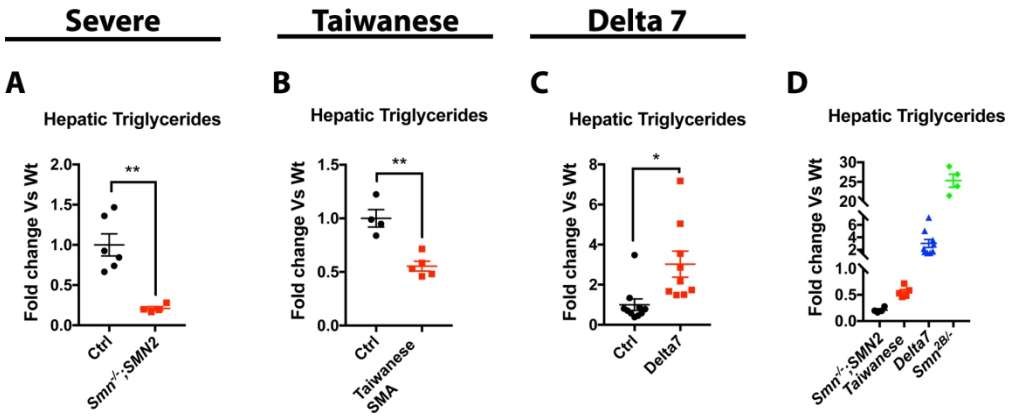
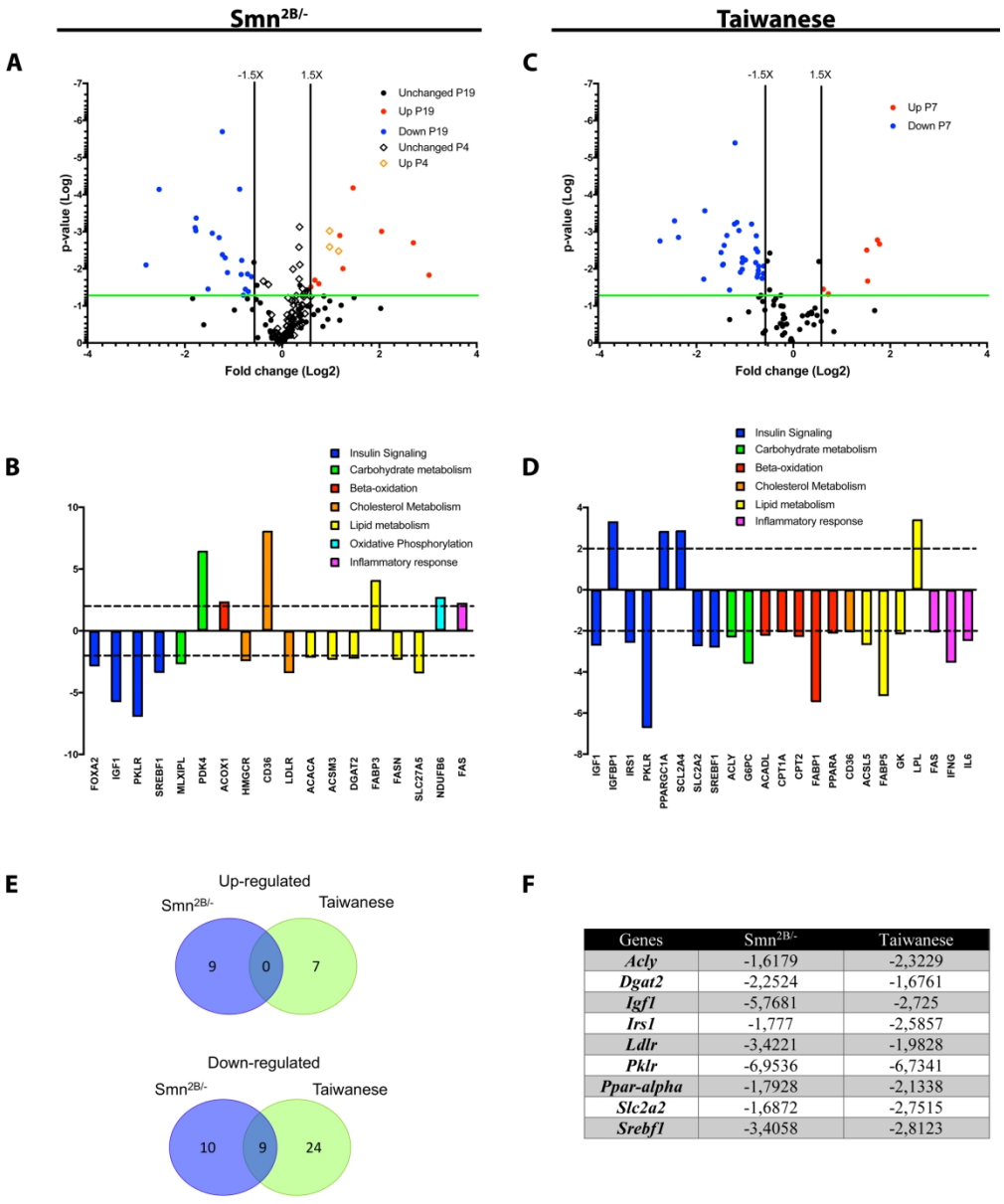


Figure 2. Hepatic triglyceride misregulation is a common feature in different SMA models at symptomatic.

155x62mm (300 x 300 DPI)



Genes	Smn$^{2B/-}$	Taiwanese
Acly	-1,6179	-2,3229
Dgat2	-2,2524	-1,6761
Igf1	-5,7681	-2,725
Irs1	-1,777	-2,5857
Ldlr	-3,4221	-1,9828
Pklr	-6,9536	-6,7341
Ppar-alpha	-1,7928	-2,1338
Slc2a2	-1,6872	-2,7515
Srebf1	-3,4058	-2,8123

Commonalities identified in expression of fatty acid metabolism genes between Taiwanese and Smn2B/- mice.

191x228mm (300 x 300 DPI)

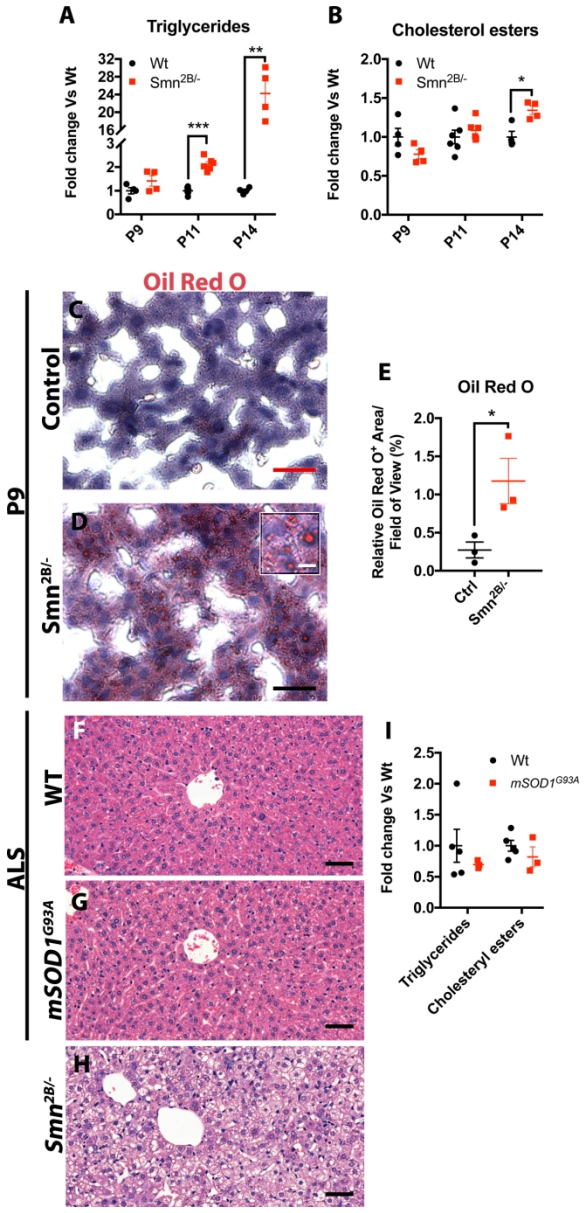


Figure 4. Fat accumulation is first observed between P9 and P11 in Smn2B/- mice and denervation is not sufficient to trigger hepatic steatosis.

112x235mm (300 x 300 DPI)

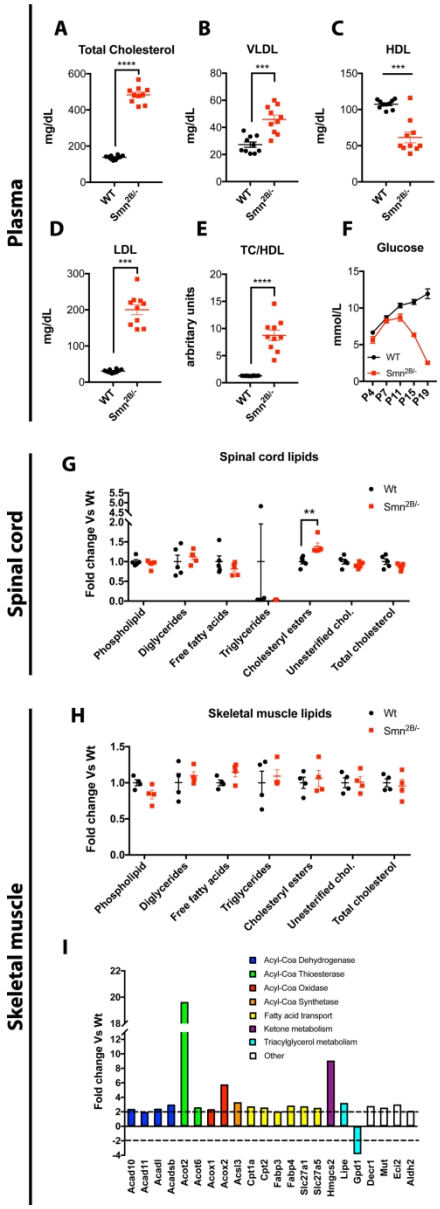


Figure 5. Smn2B/- mice display dyslipidemia and abnormal fatty acid metabolism in skeletal muscle, but not in spinal cord.

99x276mm (300 x 300 DPI)