Author's response to reviews

Title: Impaired Mitochondrial beta-Oxidation in Patients with Chronic Hepatitis C: Relation with Viral Load and Insulin Resistance

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Author's response to reviews: see over
Responses to reviewer 1

1. Improve the explanation of the rationale underlying this study: reference to

   Adult-onset type 2 citrullinemia is quite difficult to understand.

   In the light of the reviewer’s comment, and we have revised the Introduction as follows:

   “CTLN 2 is associated with mutations in the SLC25A13 gene encoding citrin, which is a component of the mitochondrial malate-aspartate shuttle. Functional defectiveness of citrin impairs not only transport of aspartate from mitochondria but that of NADH into mitochondria. This induces activation of the citrate-malate shuttle with compensatory production of acetyl CoA, which in turn stimulates fatty acid synthesis. In addition, mitochondrial accumulation of malonyl CoA in a high NADH/NAD⁺ environment suppresses fatty acid oxidation. These circumstances lead to hepatic steatosis in patients with CTLN 2 (Saheki T et al. Mol Genet Metab 2004, 81: S20-S26). Inui et al. have demonstrated that suppression of fatty acid oxidation is accompanied by impaired ketogenesis in such patients.”
2. **Define criteria for inclusion of healthy controls.**

The controls were healthy volunteers with a BMI of <25 kg/m$^2$, receiving no medication. We have stated these facts in the Patients and Methods section as follows:

“As a control group, eight volunteers (4 male and 4 female) were included (the mean age 30.5, ranging from 26 to 39 years old). All of them were healthy, with a BMI of <25 kg/m$^2$, without medication or severe disease.”

**Responses to reviewer 2**

1. *The authors showed that hepatic fatty acid oxidation was decreased in HCV patients. However, the decrease in oxidation was not significantly associated with the grade of steatosis. What is the clinical implication of the impairment of fatty acid oxidation?*
Thank you for your valuable comments. We have revised the Discussion as follows:

“Although no significant relationship between fatty acid oxidation and the grade of steatosis was demonstrated in this study (Figure 7), this issue would be worth investigating in a larger cohort of patients. HCV infection induces mitochondrial dysfunction as a result of oxidative stress, which is closely related to liver inflammation and hepatocarcinogenesis (Ivanov AV et al. Viruses 2013, 5: 439-469). Oxidative stress is associated with impairment of fatty acid oxidation, and thus impaired ketogenesis seems to represent the increased oxidative stress in CH-C patients.”

2. The authors speculated that the decrease in fatty acid oxidation was associated with insulin resistance (Discussion, 7th paragraph). This suggestion is seemingly curious, since insulin resistance generally results in the increase in serum fatty acid levels. The increased serum fatty acids may accelerate hepatic fatty acid oxidation as the authors showed in Figure 4B. The relationships between them should be discussed in more detail.

We agree with the reviewer’s suggestion. Accordingly, we have deleted the sentence
regarding the relationship between fatty acid oxidation and insulin resistance, and revised the Discussion as follows:

“In this study, a significant positive correlation was evident between the concentration of total ketone bodies and that of free fatty acids. However, in some patients with insulin resistance, the concentrations of both free fatty acids and ketone bodies were not so high. The rate of change in the concentrations of total ketone bodies was significantly lower in patients with a higher HOMA-IR value (2.5 or greater) than in those with a value of less than 2.5. Many other factors may influence the level of fatty acid. Further studies are needed to elucidate the mechanism of insulin resistance in CH-C patients.”

3. The authors discussed that “triglyceride accumulation in the hepatocytes due to the impairment of mitochondrial beta-oxidation leads to insulin resistance (Discussion, 7th paragraph).” However, it has been described that lipid accumulation in the liver does not necessarily cause the insulin resistance (Schonfeld et al. Trans Am Clin Climatol Assoc 2008; 119: 217–24). It should be discussed.
In accordance with the reviewer’s suggestion, we have deleted the inappropriate statement: “triglyceride accumulation in the hepatocytes due to the impairment of mitochondrial beta-oxidation leads to insulin resistance” (Discussion, 7th paragraph).

In relation to the second issue, further investigations will be necessary to elucidate the mechanism of insulin resistance.

4. The authors described that the increase in the core protein levels leads to the decrease in the rate of hepatic oxidation (Discussion, 5th paragraph). Because HCV replication prefers lipid rich circumstances, it can be assumed that the increase in the core protein levels are the results, not cause, of decreased fatty acid oxidation. They should be discussed.

As the reviewer suggests, the HCV core protein is located preferentially in lipid droplets and influences the replication of HCV. The previous study by Moriya et al. (Nat Med 1998,4: 1065-1067) clearly demonstrated that the core protein induces hepatic steatosis with mitochondrial dysfunction in transgenic mice expressing the HCV core. We have emphasized the above issue in the Discussion.
5. The authors evaluated hepatic fatty acid oxidation by the rate of change in ketone body concentration between 12h and 15h after the start of fasting (Methods, fasting test). Why did they measure ketone body levels at 2 times? Is this method more accurate than one point measurement? If so, it should be described.

In general, ketone bodies are not detected during periods of feeding, but after the onset of fasting, glycogen is gradually consumed and ketone bodies are produced rapidly after about 12 hours of fasting. We observed an initial increase in the rate of ketone body production between 12 and 15 hours. This was interpreted as the initial velocity of ketogenesis. We have added the following statement to the Methods section: “In general, ketone bodies are not detected during periods of feeding, but after the onset of fasting, glycogen is gradually consumed and ketone bodies are produced rapidly after about 12 hours of fasting. The rate of change in ketone body production between 12 and 15 hours represented the initial increase, and was interpreted as the initial velocity of ketogenesis.”