Response to reviewer comments

Comments to reviewer 1

We thank the reviewer for their comments.

Point 1. The reviewer comments on the generalisation of fatty acids effects, where Arachidonic acid cannot be equated with all omega-6 acids, as well as docosahexaenoic acid cannot be equated with all omega-3 acids".

Response to point 1: We have added to the text in the discussion

"However, it is important to state that not all omega 6 fatty acids are harmful/proinflammatory or omega 3 fatty acids are protective, and thus it is important not to generalise fatty acids, but to focus on specific fatty acids"

Point 2 The reviewer comments on the title.

Response to point 2: We agree and have changed the title to**:**

"High omega AA/DHA ratio induces mitochondrial dysfunction and altered lipid metabolism in human hepatoma cells"

Comments to reviewer 2

We thank the reviewer for their comments.

Point 1. The reviewer comments that limitations of the study should be discussed

Response to point 1: We have now added the following section to the discussion.

Limitations of the study

In terms of study limitations, there are a number of caveats. Firstly, measurement of hepatic phospholipid arachidonic acid (AA):eicosapentaenoic acid and AA:docosahexaenoic acid, the SCD activity which measures the conversion of fatty acids to lipids and mRNA levels of fatty acid synthase and acetyl-CoA carboxylase, would confirm the lipid changes following high omega 6:3 ratio supplementation in HepG2 cells. As we only observed ROS induced injury over a short period of time, other markers of oxidative stress such as lipid peroxides and protein carbonyls, combined with markers of endoplasmic reticulum stress, such as c-Jun N-terminal kinase, and measurement of AA metabolites such as leukotrienes would provide further weighting behind the observations. Finally, in future studies, it will be important to examine the effect of adding DHA directly to the cells after 24 hr to ascertain whether lipid accumulation can be reversed. Nutritional interventions that focus on particular omega 3 fatty acids is of interest due to the growing body of evidence showing reversal of NAFLD upon treatment with omega fatty acids.

Point 2 The reviewer comments on the format of the paper.

Response to point 2: We have addressed these issues and formatted the paper according to WJH guidelines.

Comments to reviewer 3

We thank the reviewer for their comments.

Point 1. The reviewer comments on other studies where reducing the dietary omega-6 :omega-3 did not attenuate NAFLD progression [Enos RT, et al. “Lowering the dietary omega-6: omega-3 does not hinder nonalcoholic fatty-liver disease development in a murine model.” Nutr Res. 2015 May;35(5):449-59].

Response to Point 1: This paper raises some interesting findings. However the study reported that “increasing the dietary n-6:n-3 increased the hepatic phospholipid AA:eicosapentaenoic acid and AA:docosahexaenoic acid in a dose dependent manner and mildly influenced inflammatory signalling. The method for measuring hepatic lipid accumulation was a relative qualitative procedure (i.e oil red staining) as opposed to quantitative, suggesting some limitations to the study. Clearly further work is required to evaluate the impact of omega6:3 ratio in regressing NAFLD.

We have added the following text to support the point that different ratios do/do not lead to lipid accumulation in the discussion and low ratios can reverse NAFLD:

Our findings are generally in line with a previous studies where high-fat diet with various AA:EPA and AA:DHA ratios (1:1, 5:1, 10:1 and 20:1) increased hepatic increased hepatic phospholipid AA:eicosapentaenoic acid and AA:docosahexaenoic acid in a dose dependent manner, mildly influenced inflammatory signalling, as well as key lipogenic regulators, though lowering the ratio did not prevent lipid accumulation [37] . Despite the latter study there is substantial evidence that the omega 6:3 ratio is an important contributory factor in NAFLD development. Recently NAFLD co-twin studies showed that the hepatic omega 6:3 ratio is significantly greater when liver fat >5% suggesting the impact of diet independent of genetics plays a role in NAFLD occurrence[38]. In other models of NASH where animals were fed a Western diet, omega 6 lipid concentrations were increased in hepatic membranes, whereas omega 3 lipid concentrations were reduced; inflammatory markers were also increased, and this effect was reversed when animals were given DHA[39]. Whereas with other models of fatty liver, lowering the omega 6:3 ratio attenuated gut and liver injury suggesting that a normal ratio is important in fatty liver reversal[40].

Point 2. The advantages and limitations of the study should be discussed.

Response to Point 2: We have added more detail on the advantages and limitations of the study to the discussion/conclusion

Advantages/novelty

This is the first study using high to normal omega 6:3 ratios in VL17A (HepG2 cells). These cells overexpress CYP2E1 which can metabolise AA, thus they resemble metabolic changes occurring in hepatocytes. In addition, the majority of *in vitro* NAFLD models of fatty acid treatment are based on a single 12-48 h time point to enable lipid accumulation and its subsequent detection. As far as we are aware no studies have reported a time course effect of omega fatty acids from 24 to 72 h in HepG2 cells studying lipid accumulation or ROS production, combined with lipogenic markers.

Limitations of the study

In terms of study limitations, there are a number of caveats. Firstly, measurement of hepatic phospholipid arachidonic acid (AA):eicosapentaenoic acid and AA:docosahexaenoic acid, the SCD activity which measures the conversion of fatty acids to lipids and mRNA levels of fatty acid synthase and acetyl-CoA carboxylase, would confirm the lipid changes following high omega 6:3 ratio supplementation in HepG2 cells. As we only observed ROS induced injury over a short period of time, other markers of oxidative stress such as lipid peroxides and protein carbonyls, combined with markers of endoplasmic reticulum stress, such as c-Jun N-terminal kinase, and measurement of AA metabolites such as leukotrienes would provide further weighting behind the observations. Finally, in future studies, it will be important to examine the effect of adding DHA directly to the cells after 24 hr to ascertain whether lipid accumulation can be reversed. Nutritional interventions that focus on particular omega 3 fatty acids is of interest due to the growing body of evidence showing reversal of NAFLD upon treatment with omega fatty acids.

Point 3 The format of the paper does not meet the WJH‘s requirements.

Response to Point 3: We have addressed these issues and formatted the paper according to WJH guidelines.

Comments to reviewer 4

We thank the reviewer for their comments.

Point 1. The effect of high AA:DHA ratios on intracellular triglyceride accumulation or ROS production seems transient. Other phenomena might also be transient, although the data are not shown. If so, it is unclear how these effects of high AA:DHA could contribute to the development of NAFLD. More statements are needed on the effect time.

Response to Point 1:

The following points have been included in the discussion

The majority of studies with fatty acid treatment using *in vitro* models of NAFLD are based on a single 12-48 h time point to enable lipid accumulation and its subsequent detection. Our data showed a marked increase in triglyceride accumulation after 24 h and also at 48 h, in comparison to the untreated control and healthy ratios (1:1 and 4:1) (Figure 2), which is similar to other models of NAFLD where steatosis was reported in primary hepatocytes after treatment with oleic acid[34], palmitic acid[35] or stearic acid[36]

Lipid accumulation occurred after 24 h and also at 48 h, however, after 72 h lipid concentration was normal. This suggests a biological response to high AA:DHA ratios in the short-term. Repeating the treatment again or a sustained period of omega fatty acids in the growth media, would provide further support that the response is biological rather than an acute transient effect.

After 3, 6 or 24 h we did not observe an increase in ROS production, supporting the point that incubation time is an important aspect in this model. This is similar to other models of NAFLD, where ROS production was significantly increased after 30 minutes of palmitic acid treatment[56]. Alternatively, ROS levels were lower than control cells steatotic HepG2 cells after 30 minutes incubation with DHA[57]. Other studies have shown an increase in ROS levels after a single measurement at 24 h of palmitic acid treatment[58,59]. This highlights the variance in time dependent ROS effects depending on the model used, although the net effect is increased oxidative stress.

Point 2. The authors used HepG2 cells. Since the cells derived from hepatoblastoma, it is unclear if the same effects are seen in normal hepatocytes. The authors should discuss on that point.

Response to Point 2: We have added the following studies where fatty acid treatment and subsequently steatosis was reported in hepatocytes after 24 hr treatment.

Our data showed a marked increase in triglyceride accumulation after 24 h and also at 48 h, in comparison to the untreated control and healthy ratios (1:1 and 4:1) (Figure 2), which is similar to other models of NAFLD where steatosis was reported in primary hepatocytes after treatment with oleic acid[34], palmitic acid[35] or stearic acid[36]

Conversely other studies have reported a decrease in lipid droplet levels combined with a downregulation in SCD1 & SREBP levels following DHA treatment for 12 hr in primary hepatocytes[48]

Comments to reviewer 5

Point 1. Application of arachidonic acid (AA) as omega-6 and docosahexaenoic acid (DHA) as omega-3 must introduce in clear or supply the reference(s) to support it.

Response to Point 1:

Arachidonic acid and docosahexaenoic acid were of >98% purity and acquired from Sigma-Aldrich (Gillingham, UK)

Point 2. Source of VL-17A cells and the XF Cell Mito Stress Test Kit must indicate in detail.

Response to Point 2:

VL17A cells were created by Prof Dahn Clemens. The reference was included in the methods (18) to indicate this. The XF Cell Mito Stress Test Kit was purchased from Agilent Technologies, Craven Arms, UK.

Point 3. Incubation time seems not so important in the results. Why?

Response to Point 3: We have added the following points to the discussion

The majority of studies with fatty acid treatment using in vitro models of NAFLD are based on a single 12-48 h time point to enable lipid accumulation and its subsequent detection. Our data showed a marked increase in triglyceride accumulation after 24 h and also at 48 h, in comparison to the untreated control and healthy ratios (1:1 and 4:1) (Fig.2), which is similar to other models of NAFLD where steatosis was reported in primary hepatocytes after treatment with oleic acid[34], palmitic acid[35] or stearic acid[36]

Lipid accumulation occurred after 24 h and also at 48 h, however, after 72 h lipid concentration was normal. This suggests a biological response to high AA:DHA ratios in the short-term. Repeating the treatment again or a sustained period of omega fatty acids in the growth media, would provide further support that the response is biological rather than an acute transient effect.

After 3, 6 or 24 h no increase in ROS production occurred, supporting the point that incubation time is an important aspect in this model. This is similar to other models of NAFLD, where ROS production was significantly increased after 30 minutes of palmitic acid treatment[56]. Alternatively, after 30 minutes incubation with DHA ROS levels were lower than steatotic HepG2 cells [57]. However, other studies have shown an increase in ROS levels after a single measurement at 24 h of palmitic acid treatment[58,59]. This highlights the variance in time dependent ROS effects depending on the model used, although the net effect is increased oxidative stress.

Point 4. Expression of CB2 failed to promote as CB1 in this cell line. Why?

Response to Point 4: As mentioned in the discussion, there are limited studies examining CB2 expression and its precise role in NAFLD is still being elucidated.

However, other studies have also reported a strong downregulation of CB2 gene expression in human hepatocytes following fatty acid treatment[50]. Further studies using agonists/antagonists of the CB1 and 2 receptors are required to determine their exact role in NAFLD development.

We have included this study in the discussion.

Point 5. Limitation(s) of this report may assist the unclear concerns.

Response to Point 5: We have added the following text to the discussion

In terms of study limitations, there are a number of caveats. Firstly, measurement of hepatic phospholipid arachidonic acid (AA):eicosapentaenoic acid and AA:docosahexaenoic acid, the SCD activity which measures the conversion of fatty acids to lipids, and mRNA levels of fatty acid synthase and acetyl-CoA carboxylase would confirm the lipid changes following high omega 6:3 ratio supplementation in HepG2 cells. As we only observed ROS induced injury over a short period of time, other markers of oxidative stress such as lipid peroxides and protein carbonyls, combined with markers of endoplasmic reticulum stress, such as c-Jun N-terminal kinase, and measurement of AA metabolites such as leukotrienes would provide further weighting behind the observations. The precise role of CB1 and CB2 receptor activation requires further elucidation; application of receptor agonists/antagonists would provide insight into lipogenesis under high omega ratios. Finally, it will be important to examine the effect of adding DHA directly to the cells after omega treatment to ascertain whether lipid accumulation can be reversed. Nutritional interventions that focus on particular omega 3 fatty acids is of interest due to the growing body of evidence showing reversal of NAFLD.

Point 6. There is no doubt that high omega-6: omega-3 conditions contributed to NAFLD development. However, the potential mechanism(s) fail to conduct in this report.

Response to Point 6: We have amended the conclusion to reflect the potential mechanisms

High omega 6:3 ratios of arachidonic (AA):docosahexaenoic (DHA) stimulated lipid synthesis by reducing fatty acid oxidation (decrease in PPAR alpha expression), increased CB1 expression, and also promoting the conversion of unsaturated fatty acids to saturated fatty acids via SCD1 with the net effect of lipid accumulation in human hepatoma (VL17A) cells. High ratios also led to increased oxidative stress ($P<0.01$), which is an important feature in the inflammatory state. Lipids synthesised in the liver are thus susceptible to oxidative attack by ROS; the loss of mitochondrial function possibly due to ROS or direct uncoupling effect also promotes cell death,

thus compounding the inflammatory effects. These results suggest that high omega 6:3 ratios can possibly lead to key steps in the progression from fatty liver to NASH.

Point 7. Novelty of this report needs to indicate in the conclusion.

Response to Point 7: We have added the following text to the conclusion

This is the first study using normal to high omega 6:3 ratios in VL17A (HepG2 cells). These cells overexpress CYP2E1 which can metabolise AA, thus they resemble metabolic changes occurring in hepatocytes. In addition, the majority of studies with fatty acid treatment using *in vitro* models of NAFLD are based on a single 12-48 h time point to enable lipid accumulation and its subsequent detection. As far as we are aware no studies have reported a time course effect of omega fatty acids from 24 to 72 h in HepG2 cells studying lipid accumulation or ROS production, combined with lipogenic markers.