

## **Response to the reviewer's comments**

### **Reviewer #1:**

The manuscript by Colussi et al. is an interesting report about the temporal storage and actions of nitrated FA (NO-FA).

The manuscript is well written and overall sound. The Schopfer group is well known for its experience in handling and analysing NO-FA an important prerequisite that has to be taken into account given the demanding nature of NO-FA analysis.

**We thank the reviewer for a detailed critique and the positive appreciation of the work, including a clear understanding of the analytical challenges related to measuring and characterizing these endogenous species in vivo. All revisions to the manuscript are shown in red.**

I only have one major comment.

In Figure 1 (also Figure 3) the effects of supplementation are assessed. The authors subsequently focus on phospholipids, however, I would have expected CLA to be incorporated into lipid droplets, i.e. TG and CE after supplementation. Have the authors investigated into which lipid fraction supplemented CLA is being incorporated? Also for the mice, has the incorporation of CLA into complex lipids been studied in more detail? This also stretches a bit into the involvement of ATGL as FA releasing enzyme in innate immune cells (cf the work of the Dennis group, e.g. J Leukoc Biol. 2015 Jun 24;98(5):837-850). In other words, are PL the only storage compartment? What about TG and CE.

**The reviewer brings up a good point. The distribution of CLA has been previously studied by our group and others in mouse liver and human serum lipids, showing that triglycerides and cholesterol esters are dominant storage pools of CLA<sup>1-3</sup>. The reported concentration of CLA in lipid fractions varied between studies. In mouse liver, CLA comprised of 3%, 1% and 0.1% of cholesterol esters, triglycerides, and phospholipids respectively<sup>1</sup>.**

**To better support our findings and to experimentally address the reviewer's point, we chromatographically separated the major lipid classes (phospholipids, triglycerides, and cholesterol esters) from liver, lung, and kidney tissue and measured CLA content. In agreement with previous reports, we found that CLA was mostly enriched in triglycerides in kidney and lung tissue and in phospholipids in the liver (Supplementary Figure 5, results lines 155-167). As we recognize the significance of the lipid localization for storage and signaling, we went further and established the accumulation of NO<sub>2</sub>-CLA in the specific lipid fractions. In contrast to our CLA findings, we established that for these three tissues (liver, lung, and kidney), the phospholipid fraction contained the highest level of NO<sub>2</sub>-CLA, followed by triglycerides (Figure 3A, results lines 217-223). Levels enriched in cholesterol ester were below the LOQ. This additional experimental data provides not only a distribution of the substrate CLA, but also its bioactive product NO<sub>2</sub>-CLA, showing that nitration induces a significant change in their localization.**

Minor:

Maybe nice to add the structures of NO<sub>2</sub>-12:2 etc to panel D in Figure 2.

**We agree. The structures for the electrophilic,  $\beta$ -oxidized metabolites of NO<sub>2</sub>-CLA were added to panel D in Figure 2.**

SI unit use should be checked mL or ml etc

**We updated all units across the manuscript to be consistent, using ml instead of mL.**

The letter x and the times sign should not be mixed up (very minor)

**The letter “x” was updated with the times symbol in the text (lines 85 and 512).  
Corrections are shown in red.**

How do the authors think CLA protects from vasorelaxation? By quenching NO?

**CLA cannot directly quench •NO as it doesn't add across the conjugated double bond efficiently, but the intermediate radical generated upon nitrogen dioxide (•NO<sub>2</sub>) addition reaction can react with •NO. Nonetheless, we view this as a minor pathway for •NO consumption. Based on our data, we concluded that the anti-hypotensive phenotype observed in CLA-fed mice administered LPS is primarily due to maintaining a healthier vasculature. The reduction in endothelial cell activation by NO<sub>2</sub>-CLA reduces the amplification of inflammatory signals by macrophages and other innate immune cells and contributes to maintaining vascular homeostasis and blood pressure under the •NO excess generated from iNOS during the LPS-challenge. We have previously shown that 10-NO<sub>2</sub>-OA reduces the surface expression of TLR4 and recruitment of NF- $\kappa$ B adaptor proteins to the endothelial cell membrane<sup>4</sup>. Given the similar reactivity and mechanisms of action of NO<sub>2</sub>-OA and NO<sub>2</sub>-CLA, it is likely that the endogenous nitroalkene is responsible for these vasoprotective effects.**

**We modified the discussion as follows to include these points (lines 520-532).**

In the discussion, might the microbiome not also be a valid explanation for the inconsistent human results?

**The microbiome certainly has an influence on responses to diet, LA, and CLA in humans. While the main source of CLA is dairy products, LA can also be metabolized to CLA, vaccenic acid, a precursor of CLA<sup>5</sup>. Nonetheless, it has been shown that the microbiome does not significantly impact circulating and tissue CLA levels<sup>6</sup>. The NO<sub>2</sub>-CLA formed during digestion could also be modified or metabolized by intestinal bacteria. Nonetheless, the majority of CLA and NO<sub>2</sub>-CLA absorption is expected to occur in the duodenum and jejunum, while the majority of the microbiome is located downstream in the colon<sup>7</sup>. Thus, the microbiome is expected to small impact in metabolizing CLA and NO<sub>2</sub>-CLA.**

**A short discussion was added to clarify this concept and the possible role of the microbiome (discussion lines 459-467).**

Just a thought for the discussion, but would NO not also conjugate with 7-DHC and given its high reactivity towards radicals this could be an interesting avenue for the field? See for example Ned Porters work on the oxidation of 7-DHC (also circles a but back to my comment

above).

**This is a good point, as 7-DHC is a well-known conjugated diene. Strikingly, 7-DHC is not a substrate for nitration reaction, as its conjugated diene is highly constrained, preventing addition reactions in general and, in particular, with  $\bullet\text{NO}_2$ . In contrast, 7-DHC is highly susceptible to H-atom abstraction, and  $\bullet\text{NO}_2$  could promote its oxidation<sup>8</sup>.**

**We added a sentence to explain the different reactivity of CLA and 7-DHC towards  $\bullet\text{NO}_2$ , which has been extensively studied by Dr. Porter and Dr. Lu (discussion lines 533-538).**

**Reviewer #2:**

This manuscript proposes a novel mechanism for the regulation of inflammatory responses by conjugated linoleic acid (CLA) that involves the rapid mobilization of diet-derived, preformed  $\text{NO}_2$ -CLA present in membranes. They established a mouse model to study endogenous lipid nitration reactions by using a dietary approach rich in CLA. The authors report that phospholipids in cellular membranes serve as a reservoir for the anti-inflammatory lipid signaling mediator nitro-CLA. They demonstrate that upon phospholipase A2 activation,  $\text{NO}_2$ -CLA is being released, protecting against sepsis induced cytokine release. As proof of concept, these observations were confirmed in human samples receiving lipopolysaccharide or from patients with sepsis.

The paper is an extension of work on  $\text{NO}_2$ -CLA by the authors, having high novelty and biological relevance, solid experimental approach, clear results and an interesting discussion.

**We thank the reviewer for recognizing the contribution of this work to the field.**

Minor comments: The authors postulate that after the gastric nitration of CLA, enterocytes re-esterify  $\text{NO}_2$ -CLA into triglycerides in chylomicrons. Any data to support this? They need to further clarify (probably adding a scheme) the whole metabolic pathway suggested from gastric nitration to enterocytes reesterification and transport to cellular phospholipids.

**We appreciate the comment and the opportunity to further clarify this process. We have extensively studied the absorption process of  $\text{NO}_2$ -OA in mice, rats, and dogs<sup>9,10</sup>. We recently used  $\text{NO}_2$ -OA as a prototypical nitroalkene fatty acid and investigated its absorption and pharmacokinetics, showing that it becomes esterified in enterocytes, released into the lacteal as triglycerides packed into chylomicrons, and transferred into the systemic circulation through the lymphatic system via the subclavian vein<sup>7</sup>. We found that over 95% of  $\text{NO}_2$ -OA in plasma is esterified to triglycerides, and less than 5% is bound as free acid to albumin. The proportion of  $\text{NO}_2$ -OA found to be bound to albumin is thought to derive from the spill-over effect, which corresponds to the fraction of  $\text{NO}_2$ -OA that fails to effectively transfer into the tissue and is taken back to the circulation. This is a well-known phenomenon typical for fatty acids during LPL-dependent hydrolysis in capillaries. Once in the target tissue, the  $\text{NO}_2$ -OA is trapped by rapid conjugation to Coenzyme A, which then serves as a conduit to incorporation into membranes, lipid droplets, or degradation via peroxisomal or mitochondrial oxidation. Given that  $\text{NO}_2$ -CLA and  $\text{NO}_2$ -OA are structurally closely related, we expect that both would exhibit similar absorption, transport, and tissue accumulation, but we have not performed formal pharmacokinetic studies of orally administered  $\text{NO}_2$ -CLA.**

To better clarify this mechanism, we added a scheme to Figure 2 (Panel A) to illustrate the steps involved from TG-CLA nitration in the stomach, TG-NO<sub>2</sub>-CLA absorption by enterocytes, and incorporation into chylomicrons for tissue distribution. The text was updated to reflect the addition of the scheme (results lines 169-174).

- 1 Kelley, D. S. *et al.* Contrasting effects of t10,c12- and c9,t11-conjugated linoleic acid isomers on the fatty acid profiles of mouse liver lipids. *Lipids* **39**, 135-141, doi:10.1007/s11745-004-1211-9 (2004).
- 2 Petridou, A., Mougios, V. & Sagredos, A. Supplementation with CLA: isomer incorporation into serum lipids and effect on body fat of women. *Lipids* **38**, 805-811, doi:10.1007/s11745-003-1129-2 (2003).
- 3 Burdge, G. C. *et al.* Incorporation of cis-9,trans-11 or trans-10,cis-12 conjugated linoleic acid into plasma and cellular lipids in healthy men. *J Lipid Res* **45**, 736-741, doi:10.1194/jlr.M300447-JLR200 (2004).
- 4 Villacorta, L. *et al.* Electrophilic nitro-fatty acids inhibit vascular inflammation by disrupting LPS-dependent TLR4 signalling in lipid rafts. *Cardiovasc Res* **98**, 116-124, doi:10.1093/cvr/cvt002 (2013).
- 5 Devillard, E., McIntosh, F. M., Duncan, S. H. & Wallace, R. J. Metabolism of linoleic acid by human gut bacteria: different routes for biosynthesis of conjugated linoleic acid. *J Bacteriol* **189**, 2566-2570, doi:10.1128/JB.01359-06 (2007).
- 6 Kamlage, B., Hartmann, L., Gruhl, B. & Blaut, M. Intestinal microorganisms do not supply associated gnotobiotic rats with conjugated linoleic acid. *J Nutr* **129**, 2212-2217, doi:10.1093/jn/129.12.2212 (1999).
- 7 Schopfer, F. J. *et al.* Fatty acid nitroalkenes regulate intestinal lipid absorption. *J Lipid Res* **66**, 100855, doi:10.1016/j.jlr.2025.100855 (2025).
- 8 Do, Q. *et al.* Development and Application of a Peroxyl Radical Clock Approach for Measuring Both Hydrogen-Atom Transfer and Peroxyl Radical Addition Rate Constants. *J Org Chem* **86**, 153-168, doi:10.1021/acs.joc.0c01920 (2021).
- 9 Fazzari, M. *et al.* Nitro-fatty acid pharmacokinetics in the adipose tissue compartment. *J Lipid Res* **58**, 375-385, doi:10.1194/jlr.M072058 (2017).
- 10 Schopfer, F. J., Vitturi, D. A., Jorkasky, D. K. & Freeman, B. A. Nitro-fatty acids: New drug candidates for chronic inflammatory and fibrotic diseases. *Nitric Oxide* **79**, 31-37, doi:10.1016/j.niox.2018.06.006 (2018).