Point-to-point Responses to the Reviewers' Comments

Reviewer 1

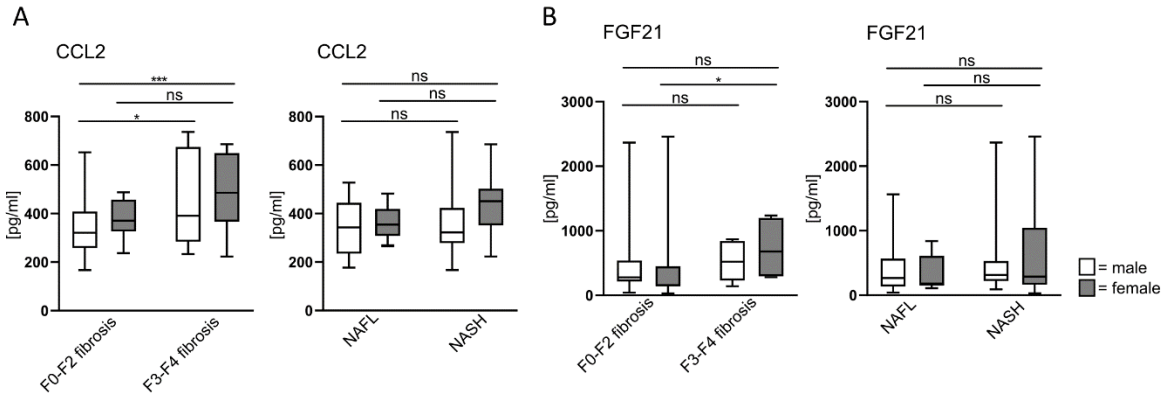
The paper by Puengel and colleagues concerns the use of CCR2 / CCR5 antagonists and FGF21 analogues in the treatment of non-aloholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). NAFLD and NASH are pathologies of great hepatological interest because, not only its incidence is constantly growing, but also because in 20/30% of cases, NASH can lead to the development of hepatocellular carcinoma or, more rarely, cholangiocarcinoma. Furthermore, currently available therapies for the treatment of NASH are dismal and it is impossible to reduce fibrotic scar, especially in the advanced stages of disease. The paper is clear and well written and the data is very interesting. Furthermore, the results are convincing and support the idea behind the work. The main results obtained by Authors are: 1. Serum concentrations of CCL2 and FGF21 correlate with different disease severity readouts. 2. Using NASH models, in vivo treatments with CCR2/5 inhibitors (CCL2 receptors), with FGF21 analogues or with a mix of them, induce a general remission of the disease and in particular of MoMF recruitment. 3. Similarly to what was seen in the mouse model of NASH (chronic damage), even in case of acute damage with CCl4, the treatment with the same compounds gives a relief of the disease. I have a few concerns about this job:

Response: We cordially thank the reviewer for his/her positive evaluation of our manuscript.

1) Since NAFLD/NASH show clear gender differences, with a higher incidence on the male population and since the Authors used only male mice for the in vivo experiments, it would be interesting to evaluate the data of figure 1 (CCL1 and FGF21 secretion and correlation with disease readouts), not only as a single group, but also splitted by gender.

   Response: We thank the reviewer for his/her expert comment. We fully agree that NAFLD is strongly influenced by gender. Male individuals predominantly show more severe stages of NAFLD such as NASH and fibrosis than female individuals during the reproductive age. However, after menopause, NAFLD occurs at a higher rate in women, supporting that estrogen is protective.

As rightfully commented by the reviewer, we added gender specific analyses as new Supplementary Figure 1:



Detailed data overview for the reviewer:

		No advanced fibrosis	Advanced fibrosis	P value
CCL2				
	Total cohort	335.3 (275.3-413.7)	432.8 (332.8-647.8)	**< 0.001**
	Men	320.8 (261-2-406.6)	390.8 (298.7-656.2)	**0.027**
	Women	371.2 (329.5-453.7)	485.8 (412.0-639.5)	0.064
FGF-21				
	Total cohort	260.6 (158.5-536.0)	674.8 (288.9-855.6)	0.067
	Men	277.7 (213.9-537.3)	521.0 (251.4-843.1)	0.394
	Women	159.8 (154-8-373.0)	679.8 (296.4-1174.5)	**0.045**
		NAFL	**NASH**	**P value**
CCL2				
	Total cohort	342.8 (267.0-400.3)	349.5 (295.3-451.2)	0.168
	Men	342.8 (234.5-420.8)	322.0 (277.8-414.5)	0.393
	Women	354.5 (319.5-400.3)	451.2 (370.3-487.8)	0.233
FGF-21				
	Total cohort	253.9 (156.4-572.3)	296.4 (194.8-562.3)	0.344
	Men	265.6 (176.0-572.7)	313.1 (222.3-516.8)	0.398
	Women	182.3 (156.4-541.4)	288.9 (157.3-927.3)	0.620

Total cohort, n = 85
Men, n = 65
Women, n = 20

However, we have to be careful interpreting the data based on our patient cohort, because the above-mentioned gender impact was also apparent in our clinical cohort. As expected from the literature (1), our cohort that was enriched for advanced disease stages displayed a lower number of female than male patients (total female patients n = 20 vs n = 65 males). In the gender specific analyses we observed a significant correlation between CCL2 serum levels and advanced fibrosis stages in male patients as well as a positive trend in female patients (p = 0.064). However, statistical power is depressed mainly based on the low numbers of cases employing the gender specific analyses and the results demonstrated

based on the whole cohort are more reliable. This is now mentioned and discussed in the revised manuscript (Section 2.1, line 123 to 128 and in the discussion line 348 to 361).

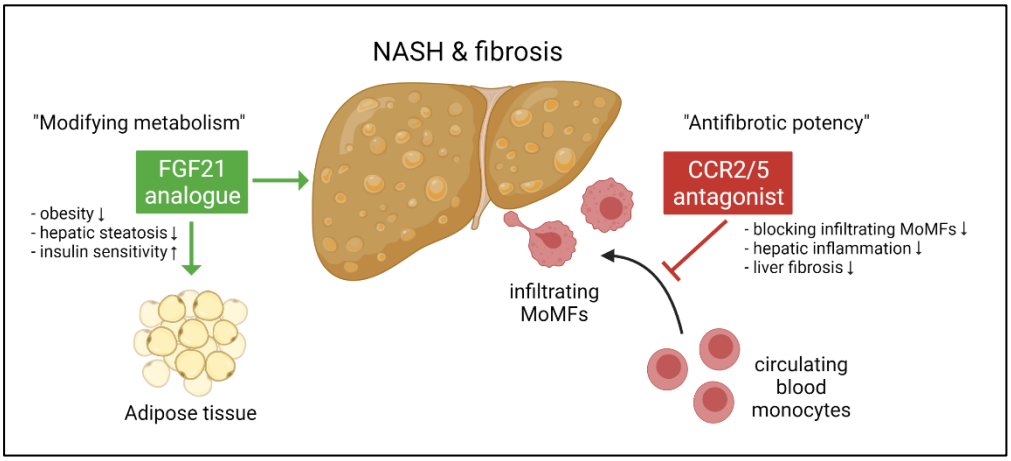
The reviewer is totally right about the comment regarding the use of male C57BL/6J mice. This is a common strategy in the field to reduce the number of mice (3R principle), since male mice are more susceptible to experimental NAFLD models and generally display a stronger, more reproducible and less variable phenotype. This is likely related to the hepato-protective functions of estrogen signaling. Therefore, employing male C57BL/6J wildtype is meant to reduce potential disease modifying factors.

2) To confirm the data in supplementary figures 1 and 2B, since an IHC was done for macrophages (f4-80), I would ask to do IHC for other immune cell populations on liver sections of control and treated mice.

Response: We would like to thank the reviewer his/her helpful suggestion to further improve the manuscript. The immune cell analyses in the current manuscript focus on macrophages (and other myeloid cells), since the CCR2/5 inhibition only affected these populations and the FGF-21 agonism did not directly impact immune cells. We provided detailed FACS analyses from circulating blood and liver tissue to demonstrate the lack of therapeutic effects on lymphocytes. Nonetheless, we agree with the reviewer that lymphocytes are involved in NAFLD/NASH and deserve detailed understanding. We are currently running large-scale multiplex immunostaining on immune cell subsets. Part of this ongoing project is being presented at this year's International Liver Congress (EASL meeting) in June 2022 in London (accepted for poster presentation, #SAT040 "Multiplex immunostaining and transcriptomic profiling identify novel immune cell markers for non-alcoholic steatohepatitis and primary sclerosing cholangitis" from our group). Since the lymphoid populations do not directly relate to the therapeutic effects of the interventions, we would rather continue with the detailed characterization of lymphoid reactions in mouse NAFLD models in the already started follow-up project (for which definite results will not be immediately available). In addition, we emphasized the cross reference in the main manuscript to Suppl. Figure 3A and B demonstrating that CCR2/5 inhibition is mainly followed by significant reduction of infiltrating MoMF without affecting lymphoid immune cell populations (line 184 to 187.

3) A graphical abstract or an explanatory drawing would be very much appreciated, in order to better clarify the mechanism that has been demonstrated.

Response: We thank the reviewer for his truly helpful comment and include a graphical abstract to the manuscript clarifying the essential underlying mechanisms and key messages:



Reference:

(1) Lonardo, A., Nascimbeni, F., Ballestri, S., Fairweather, D., Win, S., Than, T.A., Abdelmalek, M.F. and Suzuki, A. (2019), Sex Differences in Nonalcoholic Fatty Liver Disease: State of the Art and Identification of Research Gaps. Hepatology, 70: