nature portfolio

Peer Review File

Imidazole propionate is a driver and therapeutic target in atherosclerosis

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have presented interesting studies about ImP as a risk marker of CVD risk in people and as a causative factor in atherosclerosis progression in mice. Counterbalancing these results are a number of issues and questions that need to be considered:

1) Intro or Discussion: There are a number of metabolites now linked to CVD risk. One, TMAO, is included in the studies. Another prominent one is branch chain amino acids (BCAA). And, there are still others. Can the authors formulate a hierarchy or a pattern of interactions among these metabolites. In one place, they state that the association in the mouse studies was more significant because the r value (correlation to lesion size) of 0.73 for ImP was higher that the 0.5 for TMAO. Just going on absolute values, however, does not mean that these 2 r's are statistically significantly different. Is there a way to get a p value for the comparison?

2) In Fig 1, relative abundance of ImP is given- what is needed here and throughout the manuscript are absolute values. Not only would there be data to compare what is observed in mice to the clinical findings, the levels achieved in the supplementation studies and used in the in vitro studies could be assessed as to physiological relevance.

3) There very little lesion compositional analysis of the mouse plaques. It is well recognized that plaque cellular/ECM composition is a key driver of atherosclerosis progression or regression in mice, and can be independent from plaque size. In one figure, aortic digestion results on cell types/numbers are given, but this is not the same as histological sections stained with cell-type markers and markers of inflammation. In general, more comprehensive plaque analysis is needed.

4) The microbiome changes are interesting. In the mouse studies, Eubacterium and Shigella species are proposed to be important, but it seems like one may even more contributory- the increase in ImP in the HC and HCHC diets are similar, but of the two species, Fig. 1 shows that the Eubacterium abundance is more similar between the two diets than the Shigella. Please comment. Also, there does not appear to be a relationship between the microbiome in the clinical vs. mouse studies. Also, please comment/discuss.

5) "…..(i.e. 2D/3D vascular ultrasound and non-contrast computed tomography), we classified 295 participants with subclinical atherosclerosis." Is there a reference to validate the use of these methods to assign patients to these groups? This is a central issue in studies of this type.

6) PESA study patients- what about TMAO?

7) Fig 2: while the means are statistically different, there appears to be very significant overlap in the values in Fig. 2a,b,e between patient groups. How much discrimination, therefore, will plasma values afford in identifying patients at heightened risk.

8) Related to 7), one common calculation in other studies of novel risk factors, there is an analysis of how many patients would be re-classified in risk groups when combined with other risk calculators. Can this be done here?

9) Fasting glucose levels are presented in the clinical study- relationship to HbA1C would be a valuable addition.

10) "…we administered ImP in the drinking water for 8 weeks to chow-fed

ApoE-/- mice." What were the plasma levels? How do they compare with Fig 1 and to the human study?

11) Figure 3a: supplementation is done with chow and only for 8 weeks. There are 2 issues here- 8 weeks on chow will result in early lesions, raising two issues- only early lesions will be formed, whereas the goal is to study residual risk, which is typically of concern in people with advanced plaques. Secondly, there is scant information about lesion composition, a key driver of pathology.

12) Extended Data Fig. 4|"Increased ImP in circulation induces atherosclerosis and systemic inflammation." Where are the values for the circulating levels? And how do they compared to the levels in the clinical studies?

13) "scRNA-seq of aortas from 8-weeks treated mice revealed a relative increase in fibroblasts 140 (FBs), endothelial cells (ECs) and immune cells, particularly T and B cells (Fig. 3e), which was confirmed by flow cytometry analysis (Extended Data Fig. 4h)". Macrophage data are needed, especially since they are in Figure 3f.

14) Please reconcile extended Data Fig 5f of an increased response to Ifg to Fig 3f of strong downregulation of macrophage response to Ifg.

15) "…in vitro studies showed that ImP was able to activate…" Are concentrations used physiologically relevant?

16) Since ImP contains an imidazole ring we hypothesized that ImP could be sensed by ubiquitously expressed imidazoline receptors (IRs) I1R and I2R." This suggests that there cou;d be unappreciated effects of ImP on other organs that could have indirect effects on atherosclerosis. The use of a global inhibitor will not help assess this possibility.

17) Why are cholesterol levels considerably different in chow fed E-/- mice between Fig 5i and extended data Fig 3a?

18) In experiments like in Fig 4h, without ImP supplementation, the antagonist decreases atherosclerosis in the high cholesterol diet- but what are the plasma levels of ImP and how do they compare to the values that should have been reported throughout the studies?

19) Shigella bacteria go up in % in the HC diet, but the eubacterium in both HCHC and HC diets. In Fig 1f, the ImP relative levels are similarly increased by both diets- does this mean Shigella is more important?

20) "ImP and other microbiota-dependent metabolites…." Is it clear what specied of bacteria in the gut is producing ImP?

21) "We observed that administration of ImP induced local increase in transcriptional activity in endothelial cells, fibroblasts and macrophages". As noted above, this can be from effects on other organs that secrete factors that induce transcription of various genes- how can this possibility be excluded?

22) Does the receptor do anything good? In other words, is there a potential downside to blocking it?

Referee #2

(Remarks to the Author)
Mastrangelo et al. aimed to study the impact of imidazole proportionate (ImP), a microbiota-dependent metabolite on atherosclerosis. This metabolite has previously been identified as a key factor involved in insulin resistance and was associated with heart failure and all-cause mortality. Herein, the authors showed that this metabolite exerts a pro-atherogenic role. This was related to an increase in systemic and local atherosclerotic plaque inflammation, without impacting plasma cholesterol levels. This effect was related to the activation of imidazoline receptor (I1R).

While the demonstration of the pro-atherogenic role of ImP is important, the mechanistic insights are limited, which dampens the enthusiasm for this paper for Nature. The results are mainly descriptive showing an increase in inflammation without giving a clear demonstration of the underpinning mechanisms from activation of I1R by ImP to inflammation and atherosclerosis.

I have other major concerns as follows:

-atherosclerosis-related parameters are not well investigated, the authors should examine other atherosclerotic sites such as the aortic sinus, which is classically investigated in atherosclerosis-related studies.

-Also, they should examine inflammatory cell accumulation in the aortic sinus plaques

-the authors should deeply investigate the inflammatory profile of macrophages within the plaques not only facs staining of F4/80+ CD86+ as "inflammatory macrophages"

- How do they connect the results regarding pS6 (as a surrogate of mTOR activation) with the observed phenotype? Moreover, why they analyzed pS6 induction only in peritoneal macrophages and not locally within the plaques? What would be the effects of the mTOR inhibitor treatment on the phenotype in vivo?

-concerning the effects of I1R blockage on atherosclerosis progression, the authors contented to analyse systemic inflammation without investigating inflammation within the plaques.

-the authors should demonstrate the specificity of I1R antagonist. The use of I1R KO in this context would be valuable

-The connection between microbiota and ImP is missing in the experimental studies. The assessment of the specific bacteria involved in ImP-mediated proatherogenic effects would have been appreciable.

Referee #3

(Remarks to the Author)
Mastrangelo and colleagues combine human and mechanistic mouse studies to show that Imidazole Propionate (ImP) contributes to atherosclerosis and systemic inflammation without impacting cholesterol. Study also suggest that ImP could be used in combination with other risk factors to diagnose atherosclerosis at early stages and provide evidence suggesting Imidazoline receptor 1 as the potential target of ImP. While the studies provide novel epidemiological and molecular insights, there are several issues that need to be addressed.

Major:

Inclusion of a validation cohort would strengthen the manuscript.

Why does high cholesterol increases ImP in mice? This is not an expected finding as cholesterol is not a substrate for ImP production. If it is known how chol changes ImP please provide a reference. Is inflammation of the gut/availability of electron acceptors such as O2/NO3 involved in in ImP increase in response to HC diet? Is the increased cause by HC causes similar increases as detected in humans? Or when mice supplemented with ImP in drinking water?

Looks like the team has capabilities to measure ImP concentrations; mouse ImP data should be provided in concentrations so it is clear what levels the mice are exposed to in the different treatments and how this compares to conc. measured in humans?

Fig 2. Is FDR correction applied to correl analysis?

While the results with AGN192403 support the notion that ImP binds to IR1, this is not formally tested. Knockdown or cell specific (?) deletion IR1 showing reversion of phenotypes would strengthen arguments.

An novel result presented here is that ImP exacerbates athero development without changes in cholesterol in mice that have relatively low cholesterol levels (Extended Data Fig. 3), however all the results presented are either in ApoE and Ldlr ko mice. Current paradigm is that hypercholesterolemic background is necessary to induce athero in mice. Is ImP sufficient to exacerbate inflammation/exacerbate athero in wt mice?

Fig4 and Extended Fig 7. AGN192403 and ImP are provided together in drinking water. Does ImP reach the same levels when provided alone and when provided with this drug?

Previous work suggested that ImP induces activation of p38γ and subsequent activation of 2 distinct downstream signaling pathways, p62-mTORC1-S6K130 and AKT-AMPK leading to insulin resistance. Please discuss how findings presented in this study are connected with this previous work.

Other comments

“Plasma ImP concentration positively correlated with gut microbiota genera of Escherichia/Shigella and Eubacterium” What is the significance of this finding? Are bacteria in these genera known to make ImP?

“16S-based metagenomic analyses identified ImP to be positively associated with the relative abundance of Veillonella, Acidanaminococcus, Actinomyces and Megasphaera, and inversely with Erysipelotrichaceae and Coriobacteriaceae families (Fig. 2d), which were previously described to be altered in CVD subjects”

Human study is a completely different set of bacteria. Do bacteria in these families make ImP? Also, I suggest using the term 16S rRNA gene sequencing instead of 16S-based metagenomic

Figure 1 h (right) is confusing. Why same genera from different treatment groups adds 100%? Not clear what the point of this figure is

A very recent study identified ImP associated with atherosclerosis among women living with or at risk of HIV. Please cite

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
Thank you for your thoughtful responses to my many comments. The manuscript is considerably improved.

Referee #2

(Remarks to the Author)
The new submission of the revised version of the manuscript has some commendable improvements. However, the results stemming from the mouse models of atherosclerosis remain unconvincing. I asked for plaque quantifications (for all the experiments), in the aortic sinus, which is an important and classically investigated location in atherosclerosis-related studies. The authors provided unconventional quantifications as they provided a percentage (of what?) of Masson's trichrome staining whereas it was supposed to be a surface of plaques stained by Oil Red O (to visualize lipids). From the figure provided as the new extended Fig 3a, there is a very small plaque in the aortic sinus, and also in the thoracic aorta (for example extended data Fig. 4k, extended data Fig. 5o), which questions the robustness of the quantifications of such small plaques. In this regard and as the authors selected a representative picture for each result and considering the observed small plaques in the aortic sinus, they should provide the pictures of all aortas in the supplement. The small size of plaques is because the authors used mice fed CD, which had a low level of blood cholesterol levels and thus small plaques. I understood that the authors "wanted" to present ImP as an earlier marker of atherosclerosis but concluding its role in atherosclerosis based on the results showing such small plaques is not convincing. Having said that, the authors performed an experiment with HC-fed apoe-/- mice in which they only tested the effect of I1R antagonist (shown in Extended Data Fig. 8C) whereas they did not show the effects of ImP supplementation in HC-fed ldlr-/- mice. In any case, the authors should present the impact of ImP supplementation at early and late stages of atherosclerosis development and not only at a very early stage.

The second concern is regarding the in vitro experiments presented in Fig 3H and extended Fig 5J where it is unclear how the results from mouse lines (MAEC and MEF) and in vitro differentiated macrophages (BMDM) could explain the mechanisms involved in vivo? The authors stated "Moreover, RNAseq analysis showed rapid induction of proinflammatory genes in BMDMs and MEFs, but not MAECs, following ImP treatment (New Fig. 3h and New Extended Data Fig 5j), suggesting that MFs and FBs are the main targets of ImP". Again, for instance, in Fig 3H, the fact that certain mouse lines showed a rapid reactivity 1 or 2 hours after ImP stimulation did not mean that ImP had a preferential impact on these cells in vivo.

Moreover, my comment regarding the absence of the link between bacteria and ImP remains. In my opinion, this is an important aspect insofar as ImP production is dependent on microbiota and it is well known that gut microbiota has a huge impact on atherosclerosis development.

Referee #3

(Remarks to the Author)
The authors addressed adequately all my concerns. Addition of new human cohort plus additional mechanistic studies have strengthen the manuscript.

Version 3:

Reviewer comments:

Referee #5

(Remarks to the Author)
This is an interesting and comprehensive report of series of studies from animal model, in-vitro studies, to observational population cohort studies. The focus of my review is on statistical methods and two observational cohorts as instructed by the editors.

The statistical analysis methods used for the data from the PESA and IGT appears to be appropriate and the presentations of the results in the tables and test are sound. Most of the interpretations of the findings are accurate.

The major comment I have is about the ImP results in table 1a and 2a. I believe that these are raw and unadjusted data (compared with the results in the figures in which adjustments for traditional CVD risk factors were made. However, it is important to see whether the finding is statistically significant in univariate analysis before performing adjustment for potential cofounding factors.

The significance seems to be there for the PESA cohort (mean level of 28.7 in the AT group vs 26 in the Control group) as presented in table 1a, however, in the IGT cohort, there is no difference (7 vs 7). Also, not sure how the p value of 0.011 was obtained with the 7 vs 7 comparison.

One related question to this point is whether the same or similar ImP analytic tools were used in these two cohort studies. It is stated in the manuscript that ImP was confirmed by using the reference standard. The value of 28 nmol in the PESA cohort is 4 times than in the IGT cohort, although I understand that main characteristics of participants in the two cohorts are different. The difference appears to be too large. It would be helpful to explain this in the manuscript.

I have several minor observations for clarification also.

1. Line 125: PCS needs to be defined. Is it Principal Component Analysis?
2. Line 1054: do PCs mean “Principal Components”? Needs to be defined when it first appears as it does not appear to be a standard acronym.
3. Table 1a about Triglycerides:
The overall mean value is 87, while the mean for both control and AT groups is 80. Is it a typo? It should not be statistically significant with the comparison between 80 and 80.
4. Not sure how the P vale of 0.023 was obtained with the HbA1c comparison across 3 tertiles in the IGT cohort (see portion of the table 2b with mean value of 35 for all three groups):
5. About hs-CRP levels: in the PESA cohort (table 1a), the mean in the control group is 0.11 and the AT group is 0.14, which shows a marginal statistically significance, however, in the IGT cohort, the mean vale is 0.10 for the control group and 0.12 for the AT group, but the difference is highly significant. Is it because of larger sample size in the IGT cohort and the PESA cohort is under power or something else?

Version 4:

Reviewer comments:

Referee #1

(Remarks to the Author)
None

Referee #2

(Remarks to the Author)
I appreciate the authors' efforts to address my concerns regarding the quantification of small plaques. It is clear that the mouse models utilized by the authors develop very small plaques, and the rationale for using a chow diet to demonstrate that ImP increases atherosclerosis, akin to how a high-cholesterol diet elevates its levels, is well understood.

However, the main issue lies in the threshold. When mice were fed a high-cholesterol diet, they still exhibited pro-atherogenic effects of ImP, albeit with increased ImP levels in both non-supplemented and ImP-supplemented groups. It would be beneficial to include experiments conducted with a high-cholesterol diet in the supplementary figures. Alternatively, using older Apoe-/- mice, which naturally develop plaques as they age and do not require a high-cholesterol diet like Ldlr-/- mice, could provide another viable approach.

Having said that, the authors present convincing images and quantifications throughout their paper to validate the accuracy of their findings.

Referee #3

(Remarks to the Author)
The authors have adequately addressed my comments. This is a significantly improved version of the manuscript. I commend the authors for the thorough responses.

Referee #5

(Remarks to the Author)
None

POINT BY POINT RESPONSE

We appreciate that the Reviewers found our work of significant interest, and we would like to thank them for their helpful and constructive comments. We have addressed all the issues raised by the Reviewers and also discussed with the Editor in this revised version, as explained below. Changes to the main text can be tracked in the marked copy. In the response, we refer to many new figures corresponding to newly added experiments in the manuscript (highlighted as new). We also discuss some figures for the Reviewers, which presence in the manuscript is not strictly necessary, in our opinion. However, if the Reviewers or the Editor think that we should include any of these figures for the Reviewers in the manuscript, we are happy to do so.

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have presented interesting studies about ImP as a risk marker of CVD risk in people and as a causative factor in atherosclerosis progression in mice. Counterbalancing these results are a number of issues and questions that need to be considered:

1) Intro or Discussion: There are a number of metabolites now linked to CVD risk. One, TMAO, is included in the studies. Another prominent one is branch chain amino acids (BCAA). And, there are still others. Can the authors formulate a hierarchy or a pattern of interactions among these metabolites.

We agree with the Reviewer's comment regarding the presence of several microbially produced/regulated metabolites in host bloodstream. To bench-mark our model, we specifically decided to focus on TMAO, the most established metabolite associated with CVD produced by the gut microbiota. However, following the Reviewer's suggestion but also taking into account the strict word limit imposed by the journal, we have added some known and additional recently reported microbially produced metabolites to the introduction and discussion, including various amino acid-derived metabolites (https://doi.org/10.1093/eurheartj/ehad333) such as phenylacetylglutamine (PAGln), that have been associated with CVD (https://doi.org/10.1016/j.cell.2020.02.016, https://doi.org/10.1161/CIRCRESAHA.120.316242) (lines 70ff and 242ff).

Regarding the BCAAs, although primarily known for their association with type 2 diabetes and comorbidities, they have been recently associated with CVDs. We assessed their relative abundance in our untargeted metabolomics experiment, finding that the microbiota does not significantly modulate the relative abundance of BCAAs (**Fig. 1 for the Reviewers**). This is consistent with BCAAs not being *bona fide* microbially produced metabolites; rather, they are essential nutrients that can also be obtained from the diet (https://doi.org/10.1093/ajcn/nqz025). Therefore, while we acknowledge the potential association of these additional metabolites (BCAAs and other amino acid-derived

metabolites) with imidazole propionate (ImP), we believe that introducing hierarchies or pattern of interactions among them would dilute the central message of our work, which focuses on ImP as a new marker of early atherosclerosis and ImP sensing by I1R as a potential target for therapy.

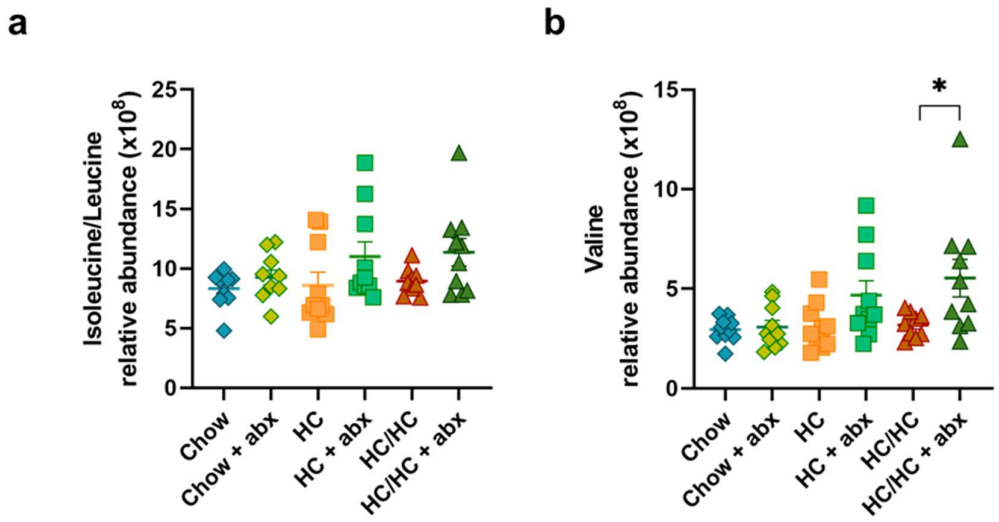


Fig. 1. for the Reviewers. Relative abundance of branched-chained amino acids (BBCAs), including isoleucine /leucine (**a**) and valine (**b**) in plasma from *ApoE*$^{-/-}$ mice fed chow, high cholesterol (HC) or HC and high choline (HC/HC) diets in the presence or not of broad spectrum mix of antibiotics (abx). Plasma samples were collected at culling (n=10 per group) and the metabolites measured by untargeted LC(HILIC)-MS ESI+. Individual data and arithmetic mean ± SEM of each group from two pooled independent experiments is shown. One-way ANOVA with Tukey post-hoc correction. ∗ $p < 0.05$ vs. control (the same diet without abx)

In one place, they state that the association in the mouse studies was more significant because the r value (correlation to lesion size) of 0.73 for ImP was higher that the 0.5 for TMAO. Just going on absolute values, however, does not mean that these 2 r's are statistically significantly different. Is there a way to get a p value for the comparison?

Following the Reviewer's suggestion, we have performed the Fisher's z-transformation test to assess the significance of the difference between the two correlation coefficients, specifically the correlation between ImP or TMAO and lesion size. The statistical analysis showed that the difference between the two coefficients is not statistically significant:

R_{ImP}= 0.73 & R_{TMAO}=0.5
R_difference =0.23 (-0.1599 – 0.72448)
Z= 1.37336 p= 0.16946

Thanks for pointing this out. We have deleted the sentence that suggested a higher correlation of ImP compared with TMAO and we simply indicate that both correlate with atherosclerosis lesion (lines 83ff).

2) In Fig 1, relative abundance of ImP is given- what is needed here and throughout the manuscript are absolute values. Not only would there be data to compare what is observed in mice to the clinical findings, the levels achieved in the supplementation studies and used in the in vitro studies could be assessed as to physiological relevance.

While we agree with the Reviewer's comment regarding the relevance of providing absolute values for the concentration of ImP in the bloodstream, we are unable to provide those values in reference to the experiment that was performed in Fig. 1, since the plasma sample available was used up to carry out the untargeted metabolomics experiment. Fortunately, we can provide ImP concentration in plasma samples that we collected from the subsequent experiments using the same chow and high cholesterol (HC) diets. We found increased bloodstream levels of ImP in *ApoE*$^{-/-}$ mice fed a HC diet in comparison with the chow diet (**Fig. 2 for the Reviewers**), confirming the results from our untargeted experiment shown in Fig. 1.

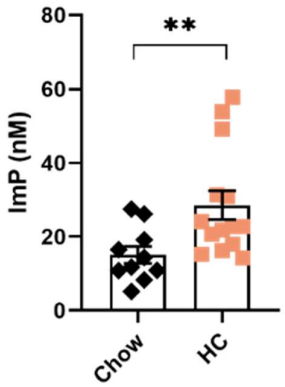


Fig. 2 for the Reviewers. Plasma imidazole propionate (ImP) from *ApoE*$^{-/-}$ mice fed chow or high cholesterol (HC) diets for 8 weeks. Individual data and mean ± SEM from two pooled independent experiments. Welch test ** $p < 0.01$.

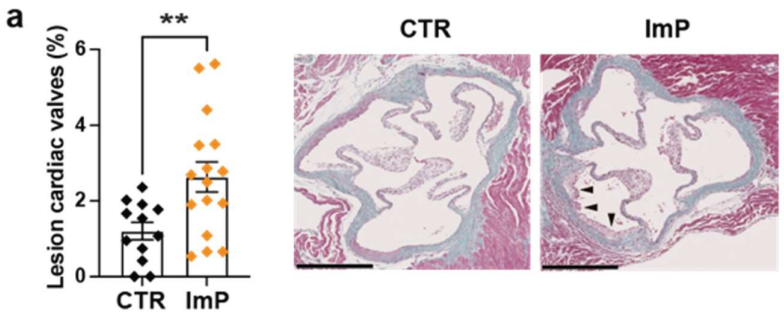
Regarding ImP quantification in plasma from mice fed a high cholesterol-high choline diet (HC/HC), we cannot provide its actual concentration. However, we would like to emphasize that this diet induces an increase in ImP levels similar to the effect seen for the HC diet (Fig. 1f), with whom it shares a similar composition: HC is 10% fat and 0.75% cholesterol (SSNIFF S9167-E011), and HC/HC is actually HC supplemented with 1% of choline (SSNIFF S9167-E016). Thus, while formal validation of the result in the HC/HC diet would require quantifying ImP in mice fed that specific diet, we believe that the similar composition of the two HC diets and the fact that choline is not a known precursor of ImP would suggest a similar increase in ImP in *ApoE*$^{-/-}$ mice fed a HC/HC diet

to that found in mice fed the HC diet, as we have seen in our untargeted metabolomics analysis (Fig. 1f).

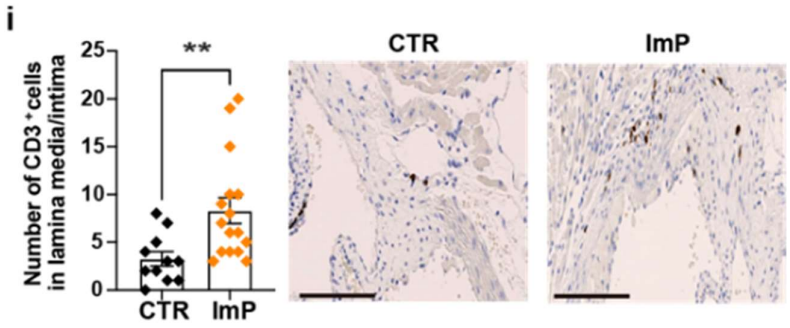
Other aspects commented by the Reviewer, namely absolute values for ImP in other experiments, such as supplementation studies, and *in vitro* studies, have also been addressed experimentally and are explained in detail in the response to specific points 10, 15, and 18.

3) There very little lesion compositional analysis of the mouse plaques. It is well recognized that plaque cellular/ECM composition is a key driver of atherosclerosis progression or regression in mice, and can be independent from plaque size. In one figure, aortic digestion results on cell types/numbers are given, but this is not the same as histological sections stained with cell-type markers and markers of inflammation. In general, more comprehensive plaque analysis is needed.

We thank the Reviewer for this suggestion. We agree that alterations in the composition of atherosclerotic plaques are as crucial as their extent. In line with the Reviewer's recommendation, we assessed both plaque extension and its composition within the cardiac valves, aiming for a more comprehensive characterization of the atherosclerotic plaque. Treatment of chow-fed *ApoE*$^{-/-}$ mice with ImP for 8 weeks induced the development of increased atherosclerotic plaques in the cardiac valves, accompanied by increased infiltration of T lymphocytes in the media and intima layers compared with control mice (**New Extended Data Fig. 3a and New Extended Data Fig. 4i)**. Furthermore, we analysed structural changes in the vascular wall using Movat's pentachrome staining, as well as vascular smooth muscle cells through SMA2 labelling, without observing any changes associated to ImP administration *in vivo*. The plaque induced by ImP administration was incipient, with low macrophage infiltration and no detected caspase-3 staining or necrotic core (**Fig. 3 for the Reviewers, below**).



New Extended Data Fig. 3a. *ApoE*$^{-/-}$ mice were administered ImP (ImP) or not (CTR) for 8 weeks. Masson's trichrome staining of the aortic valves performed under the indicated treatments. Left: Quantification of atherosclerotic lesions as percentage of total valve area. Right: Representative images. n=12-16. Individual data and mean ± SEM from at least two pooled independent experiments. Unpaired Student's t test. ** $p < 0.01$.



New Fig Extended Data 4i. *ApoE*$^{-/-}$ mice were administered ImP (ImP) or not (CTR) for 8 weeks. Left: representative images of CD3 staining in aortic valves. Right: quantification of CD3^{+} cells in aortic valves as numbers n=11-16. ** $p < 0.01$ by Unpaired Student's t test.

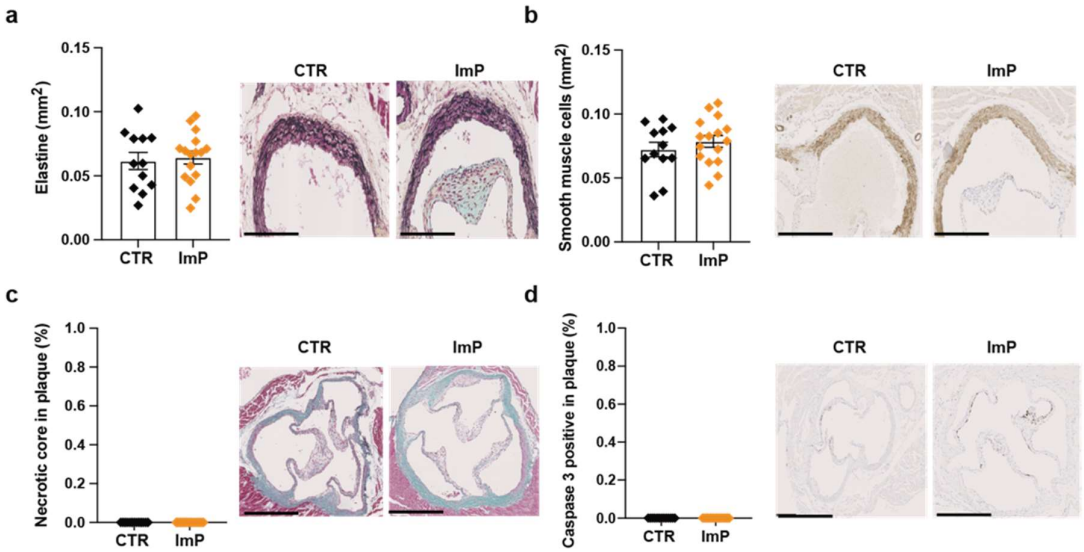
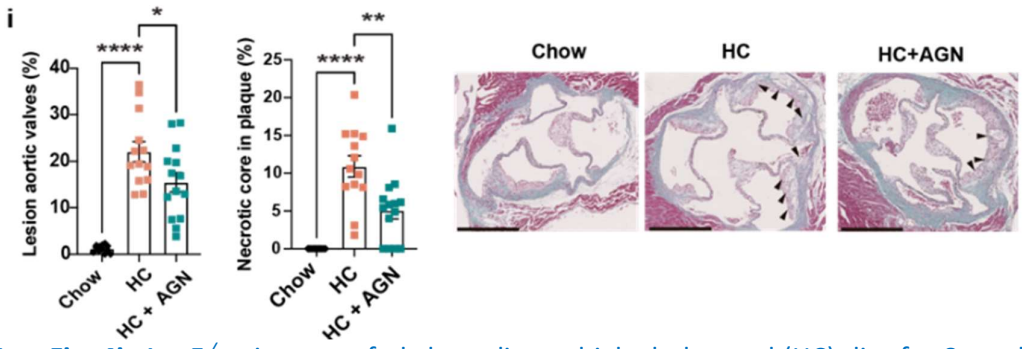


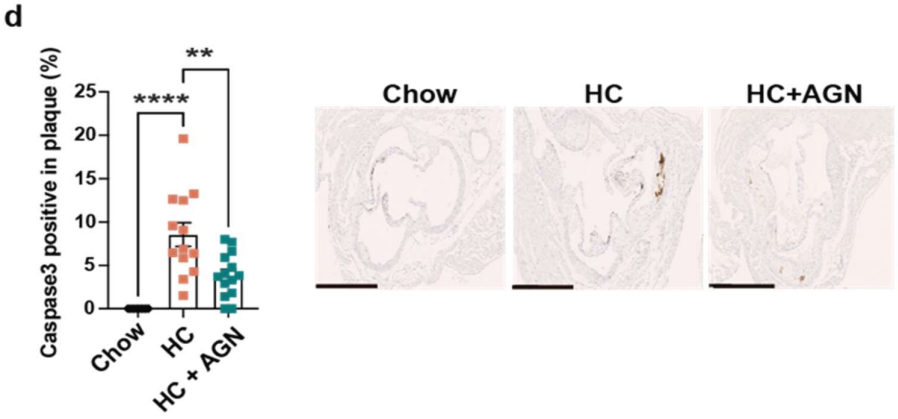
Fig. 3. for the Reviewers. *ApoE*$^{-/-}$ mice were fed a chow diet for 8 weeks and treated or not (CTR) with ImP in the drinking water. **a**, Integrity of elastin fibers, quantified as area of elastin with Movat's pentachrome staining. Black areas were used to quantified Elastin fibers, showing quantification (left) and representative images (right), n=12-16. **b**, Integrity of smooth muscle cells (SMC) quantified as area of SMC using SMA antibody, showing quantification (left) and representative images (right), n=12-16. **c**, Percentage of necrotic core area inside the total area plaque, quantified as non-Masson's trichrome staining areas in cardiac plaque showing quantification (left) and representative images (right), n=13-17. **d,** Quantification of Caspase 3 positive stain in cardiac valves lesion showing quantification (left) and representative images (right), n=13-17. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction.

Moreover, we have now analysed the effect of the AGN192403 treatment on the changes in the composition and stability of the plaque upon HC diet-induced

atherosclerosis. AGN192403 treatment (from week 5 to 8) in *ApoE*$^{-/-}$ mice fed HC diet for 8 weeks effectively reduced the percentage of plaque formation in the aortic valves compared with untreated mice (**New Fig. 4i**). Additionally, AGN192403 administration resulted in lower expression of caspase 3 along with a decrease in the necrosis area within the atherosclerotic plaque (**New Fig. 4i and new Extended Data Fig. 8d**), which is associated to reduced plaque complexity in mice (https://doi.org/10.1038/s41467-023-36614-w; https://doi.org/10.1161/atvbaha.122.318177).



New Fig. 4i, *ApoE*$^{-/-}$ mice were fed chow diet or high cholesterol (HC) diet for 8 weeks. At 4 weeks post diet initiation, AGN192403 was administered (AGN) or not in the drinking water to mice fed HC diet until week 8, followed by sacrifice and analysis. **i**, Masson's trichrome staining of aortic valves. Quantification of lesion in aortic valves (left), necrotic core area (middle) and representative images (right). Arrowheads indicate necrotic areas. Bar size= 500µm. n=12-14. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.001$.



New Extended Data Fig. 8d, *ApoE*$^{-/-}$ mice were fed chow diet or high cholesterol (HC) diet for 8 weeks. At 4 weeks post diet initiation, AGN192403 was administered (AGN) or not in the drinking water to mice fed HC diet until week 8, followed by sacrifice and analysis. **d,** Caspase 3 staining of aortic valves. Quantification of caspase 3+ (left) and representative images of Caspase 3 staining (right) Bar size= 500µm. n=12-14. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. ** $p < 0.01$; **** $p < 0.001$.

4) The microbiome changes are interesting. In the mouse studies, Eubacterium and Shigella species are proposed to be important, but it seems like one may even more contributory- the increase in ImP in the HC and HCHC diets are similar, but of the two species, Fig. 1 shows that the Eubacterium abundance is more similar between the two diets than the Shigella. Please comment. Also, there does not appear to be a relationship between the microbiome in the clinical vs. mouse studies. Also, please comment/discuss.

We thank the Reviewer for this insightful comment. The Reviewer is correct that the Eubacterium is more consistently increased in both HC and HC/HC diets. In agreement, it is also more strongly correlated with ImP. However, since we previously showed that one amino acid difference in the active site of urocanate reductase can shift specificity of the enzyme, we do not think we can extrapolate the data further (https://doi.org/10.1038/s41467-021-21548-y). However, the data are consistent with the association of ImP production with reduced diversity in the gut microbiota (https://doi.org/10.1038/s41587-019-0233-9 and https://doi.org/10.1038/s41467-020-19589-w). This suggests that the altered microbial ecosystem associated with atherosclerosis in our study produces increased amounts of ImP that is absorbed from the gut and contributes to atherosclerosis in the host.
It is not surprising that the gut microbiota differs from mice and humans since this is well known in the literature (https://doi.org/10.1194/jlr.M072819) and this is also the reason why we do not compare the datasets directly. Moreover, the species contributing to ImP production could be different in mouse and human.

5) "…..(i.e. 2D/3D vascular ultrasound and non-contrast computed tomography), we classified 295 participants with subclinical atherosclerosis." Is there a reference to validate the use of these methods to assign patients to these groups? This is a central issue in studies of this type.

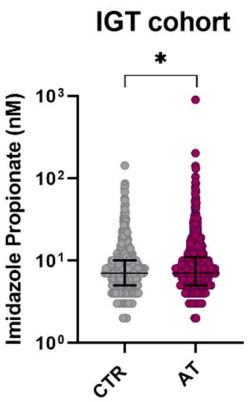
Thanks for raising this issue. The categorization of subjects as having subclinical (i.e. asymptomatic) atherosclerosis or not, based on peripheral arteries (carotid and/or femoral ultrasound) and/or coronary calcification on non-contrast computed tomography, is a well-accepted methodology. Besides our cohort (PESA), this methodology has been used in cohort studies such as:

- Bioimage (https://doi.org/10.1016/j.jacc.2015.01.017)
- AWHS (https://doi.org/10.1016/j.jacc.2015.12.056)
- Cyprus (https://doi.org/10.1016/j.jacc.2022.03.352)
- YFS (https://doi.org/10.1016/j.atherosclerosis.2019.11.029), among others.

There is a clinical practice guideline by the American Society of Echocardiography for the use of vascular ultrasound to identify and quantify subclinical atherosclerosis (https://doi.org/10.1016/j.echo.2020.04.021). Finally, the European Society of Cardiology (ESC) cardiovascular disease prevention clinical practice guideline acknowledges the use of these methodologies to diagnose subclinical atherosclerosis and emphasizes that this condition increases the risk for future cardiovascular mortality (https://doi.org/10.1093/eurheartj/ehab484).

6) PESA study patients- what about TMAO?

To provide the Reviewer some insights into the abundance of TMAO in an early atherosclerosis healthy volunteer cohort similar to PESA, we have analyzed this data from an untargeted metabolomics-based experiment of the IGT cohort (https://doi.org/10.1016/j.ahj.2024.01.011). This cohort shares similarities with our PESA cohort and has been used to validate our results on ImP as a biomarker of subclinical atherosclerosis **(New Fig. 2a)**. Unlike ImP, TMAO levels in plasma remain unchanged between volunteers with and without early atherosclerosis in this cohort (**Fig. 4 for the Reviewers**). While this finding supports the relevance of ImP as a specific marker of early atherosclerosis, we feel that the comparison of ImP and TMAO as markers for atherosclerosis at different stages of the disease (ImP being a marker for early subclinical atherosclerosis and, particularly, active plaque) would require a dedicated and separate study and further research that would involve considerable time and cost and is out of the scope of the present manuscript. However, if the Reviewer or Editor think that it is relevant to include this result in the manuscript, we will be happy to do it.



New Fig. 2a: Plasma levels of imidazole propionate (ImP) in healthy subjects (CTR, n=838) and subjects with subclinical atherosclerosis (AT, n=1006) from the IGT cohort. LCMS-based targeted metabolomics was used to determine the concentration of ImP in plasma. Arithmetic mean ± SEM of each group is shown. Mann–Whitney U test. *p<0.005.

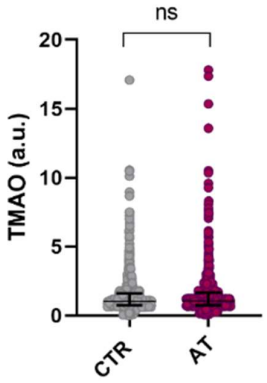


Fig. 4 for the Reviewers: Plasma levels of TMAO in healthy subjects (CTR, n=838) and subjects with subclinical atherosclerosis (AT, n=1006) from the IGT cohort. LCMS-based untargeted metabolomics was used to determine the relative abundance of TMAO. Arithmetic mean ± SEM of each group is shown. Not significant (ns) by Mann–Whitney U test.

7) Fig 2: while the means are statistically different, there appears to be very significant overlap in the values in Fig. 2a,b,e between patient groups. How much discrimination, therefore, will plasma values afford in identifying patients at heightened risk.

8) Related to 7), one common calculation in other studies of novel risk factors, there is an analysis of how many patients would be re-classified in risk groups when combined with other risk calculators. Can this be done here?

We thank the Reviewer for the comment. In order to clarify these points (7 and 8), we have performed three types of statistical analyses: 1. Assessment of the relative risk using ImP or other clinically recommended markers 2. Comparison of the areas under the ROC curves obtained for ImP, LDL-C, ImP-panel based on LASSO regression model (i.e. PANEL) and SCORE2, and 3. Net reclassification improvement (NRI) analysis, as suggested by the Reviewer.

1. The relative risk (RR) is used to quantify the relationship between an exposure and an outcome. Specifically, it describes the likelihood of occurrence of an event after exposure to a risk variable (i.e. maker levels higher than a certain cutoff value) in comparison with the likelihood of its occurrence in a control or reference group. Using SPSS, we assessed the RR of ImP as well as of the gold-standard markers used in the clinics (LDL-C, total cholesterol, and HDL-C). In order to calculate the RRs, we used the cutoff values proposed in our manuscript for ImP (41nM) and the clinically recommended for LDL-C (160mg/dL), total cholesterol (240mg/dL) and HDL-C (40mg/dL). As shown below, subjects with ImP levels higher than its cutoff are 19% more likely to have subclinical atherosclerosis. In contrast, considering the cutoffs for LDL-C, total cholesterol and HDL-C, the percentages drop to 7%, 7% and 2%, respectively. Moreover, unlike the other markers, only in the case of ImP, the RR is statistically significant (RR >1 and the confidence interval (CI) does not include 1).

Risk Estimate for ImP	**Value**	**95% CI**	
		Lower	**Upper**
Odds Ratio for ImP (CTR/AT)	0.452	0.251	0.814
For CTR	0.539	0.333	0.873
For AT	1.194	1.067	1.337
N of valid cases	400		

Risk Estimate for LDL-C	**Value**	**95% CI**	
		Lower	**Upper**
Odds Ratio for LDL-C (CTR/AT)	0.766	0.424	1.386
For CTR	0.818	0.519	1.290
For AT	1.068	0.931	1.226
N of valid cases	398		

Risk Estimate for Total Cholesterol	**Value**	**95% CI**	
		Lower	**Upper**

Odds Ratio for Total Cholesterol (CTR/AT)	0.777	0.496	1.217
For CTR	0.831	0.599	1.154
For AT	1.07	0.947	1.208
N of valid cases	399		

Risk Estimate for HDL-C	**Value**	**95% CI**	
		Lower	**Upper**
Odds Ratio for Total Cholesterol (CTR/AT)	0.918	0.483	1.744
For CTR	0.939	0.59	1.496
For AT	1.023	0.857	1.221
N of valid cases	399		

2. Pairwise analysis of ROC curves was conducted using the Hanley and McNeil method (https://doi.org/10.1148/radiology.148.3.6878708). Specifically, when resolving the equation described by the Hanley and McNeil method to compare the ROC of ImP and LDL-C, the Z value obtained was 1.047, pointing out no significant difference between the two ROC curves. On the contrary, the Z value obtained for the ROC difference between our PANEL and SCORE2 was 2.247 (p-value=0.0246), underlying a significant difference. These results were further confirmed using the bootstrap test to compare the AUCs proposed by Robin et al (https://doi.org/10.1186/1471-2105-12-77) (**Fig. 5 for the Reviewers and New Extended Data Fig. 2e)**. As demonstrated below, we obtained a Z of 1.1068 (p-value=0.2684) for the comparison between the AUCs calculated for ImP and LDL-C using bootstrapping. On the contrary, we obtained a Z of 2.299 (p-value=0.0215) for the comparison between the AUCs calculated for PANEL and SCORE2 models using bootstrapping. This finding highlights the improved capacity of our PANEL of markers, including ImP, when compared to the standard SCORE2.

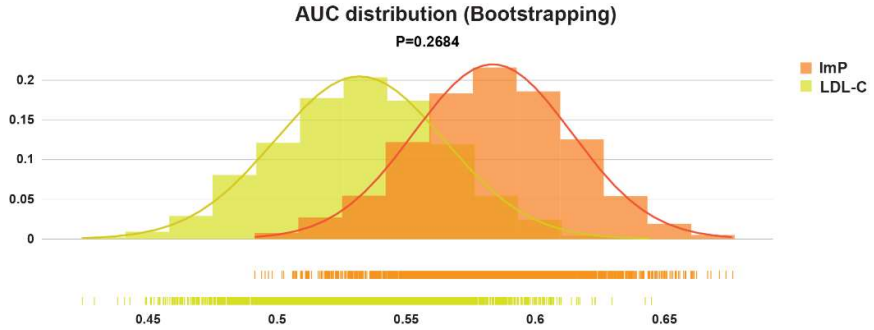
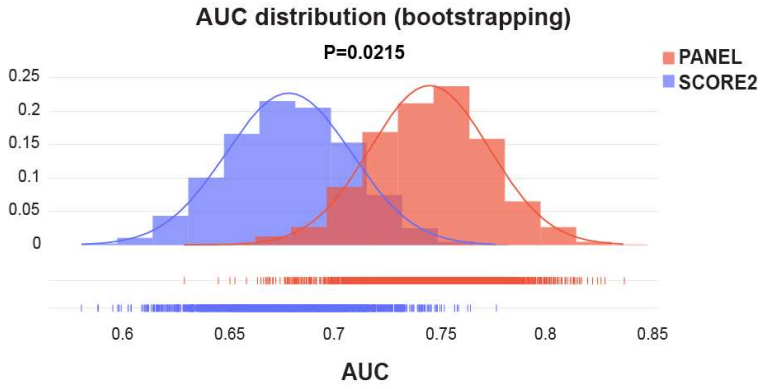


Fig. 5 for the Reviewers. Comparison between the AUC values obtained for ImP and LDL-C using the bootstrap method.



New Extended Data Fig. 2e. Comparison between the AUC values obtained for the ImP-panel based LASSO regression model and SCORE2 model using the bootstrap method. Variables in the PANEL are ImP, family history of CVDs, hypertension, active smoking, haemoglobin, creatinine and familial hypercholesterolemia.

3. We performed a net reclassification improvement (NRI) analysis to assess the reclassification of patients in risk groups when our PANEL of markers is used, compared to the SCORE2 (https://doi.org/10.1002/sim.4085). The NRI was computed by defining two categories: control (i.e. CRT) and subclinical atherosclerosis (i.e. AT). To classify each observation (using either PANEL or SCORE2) we used a threshold value that maximizes the difference between True Positive Rate (TPR) and False Positive Rate (FPR), derived from the ROC curve. As depicted below, the NRI is equal to 0.1484 (95% CI: 0.0211-0.2758), indicating an improvement of the classification of volunteers in the risk group when the PANEL instead of the SCORE2 is used.

NRI= 0.1484, 95% CI (0.0211-0.2758) p-value: 0.022

AT		SCORE2	
		False (CTR)	True (AT)
Panel	False (CTR)	39	43
	True (AT)	56	153

CTR		SCORE2	
		False (CTR)	True (AT)
Panel	False (CTR)	51	23
	True (AT)	12	20

In summary, all three statistical analyses consistently demonstrated the heightened discriminatory capacity of ImP and the PANEL of markers that we proposed in the context of subclinical atherosclerosis. To avoid overloading the article with excessive data and considering that the ROC curve analysis is the standard methodology for evaluating the diagnostic power of a biomarker, we have just included **New Extended Data Fig. 2e** in the manuscript, corresponding to the pairwise comparison analysis of ROC curves and showing the improved discriminatory capacity to diagnose early atherosclerosis of our PANEL of markers, including ImP, when compared to the standard SCORE2.

9) Fasting glucose levels are presented in the clinical study- relationship to HbA1C would be a valuable addition.

We appreciate the Reviewer's comment. We would like to clarify that the relationship to HbA1C is already included in the correlation analysis (**Fig. 2d**) and reported in the figure as glycohemoglobin. In order to avoid confusion, we have now added the HbA1C abbreviation in the figure.

10) "…we administered ImP in the drinking water for 8 weeks to chow-fed ApoE-/- mice." What were the plasma levels? How do they compare with Fig 1 and to the human study?

Following the Reviewer's request, we quantified the concentration of ImP in plasma samples from chow-fed *ApoE*$^{-/-}$ mice supplemented with ImP in the drinking water for 8 weeks. Chow-fed *ApoE*$^{-/-}$ mice showed an 8-fold increase in ImP plasmatic levels when this metabolite was administered in the drinking water (400µg/mouse/day) from 16.3±3.8nM in the untreated group to 132.60±32.47nM in the group treated with ImP (**Fig. 6 for the Reviewers**). Of note, the range of ImP concentration in the plasma upon ImP administration was 13.42-340.00nM, similar to the range found in the plasma of human healthy volunteers with atherosclerosis in the PESA cohort (1.26-266nM) and in the IGT cohort (2-899nM) (**New Fig. 2a**). In addition, in previous works we have found a range of blood concentration of ImP in humans up to 1µM (https://doi.org/10.1016/j.jchf.2023.03.008). These results suggest that the ImP concentration reached in mouse plasma upon administration of ImP in drinking water (13.42-340.00nM) is in the physiological range and similar to the concentration of ImP in human subjects showing a significant increase in ImP concentration in plasma associated to atherosclerosis (beyond 41nM).

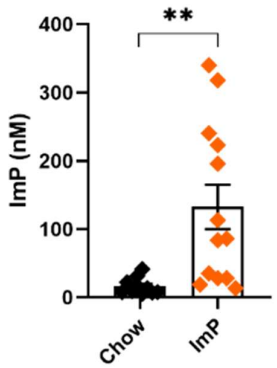


Fig. 6 for the Reviewers. Plasma imidazole propionate (ImP) from chow-fed *ApoE*$^{-/-}$ mice administered or not ImP (400µg/mouse/day) in the drinking water for 8 weeks. Individual data and mean ± SEM from two pooled independent experiments. Welch test ** $p < 0.01$.

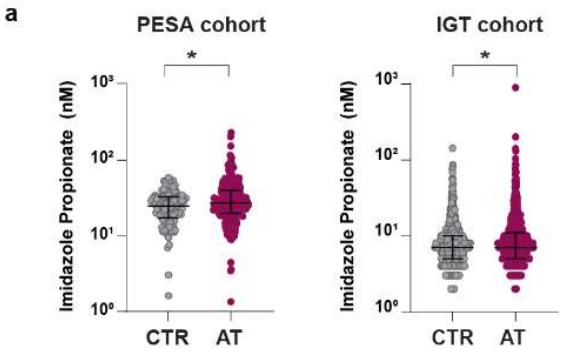


Fig. 2a: Plasma concentration of the imidazole propionate (ImP) in healthy subjects (CTR) and subjects with subclinical atherosclerosis (AT) from the PESA cohort (left, CTR n=105, AT n=295) and the IGT cohort (right, CTR n=838, AT n=1006). Mann–Whitney U test. *p<0.05.

11) Figure 3a: supplementation is done with chow and only for 8 weeks. There are 2 issues here- 8 weeks on chow will result in early lesions, raising two issues- only early lesions will be formed, whereas the goal is to study residual risk, which is typically of concern in people with advanced plaques.

Secondly, there is scant information about lesion composition, a key driver of pathology.

We would like to emphasize that the main objective of our work is to study and propose ImP as a new marker of early atherosclerosis, rather than investigating the residual risks that are generally associated with a more advanced stage of the disease. For this reason, we decided to quantify ImP in a seemingly healthy population where atherosclerosis is detectable only through advanced imaging techniques not readily available in clinics (Fig. 2). This approach enabled us to propose ImP and the ImP-based PANEL as early markers of subclinical atherosclerosis. Thus, in our subsequent mechanistic studies on ImP, we focused on models that could mirror the early formation of atherosclerotic lesion (i.e. chow-fed *ApoE*$^{-/-}$ mice for only 8 weeks with or without ImP administration, Fig. 3). Our studies revealed that ImP induced early atherosclerosis independently of circulating lipids, as the cholesterol concentration upon ImP administration did not change. This later finding highlights the potential of ImP as a novel factor in the disease aetiology that is distinct from the well-known cholesterol metabolism.

Furthermore, based on the recommendation here and in point 3, we have analyzed the composition of atheroma plaques in the different models. These results are already discussed above in response to point 3.

On the other hand, we agree with the Reviewer that, in addition to serving as an early indicator of atherosclerosis, ImP could be a potential biomarker for later cardiovascular

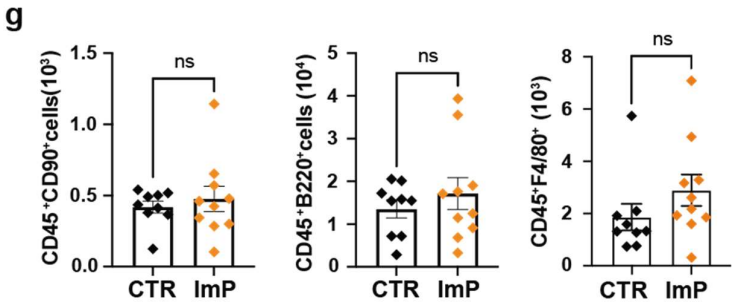
events, thereby enhancing the relevance of our therapeutic approach. This perspective is supported by a recent publication demonstrating the association of ImP with heart failure and all-cause mortality (https://doi.org/10.1016/j.jchf.2023.03.008), but its exploration would require a separate study and analysis of different cohorts in a late stage of the disease.

12) Extended Data Fig. 4|"Increased ImP in circulation induces atherosclerosis and systemic inflammation." Where are the values for the circulating levels? And how do they compared to the levels in the clinical studies?

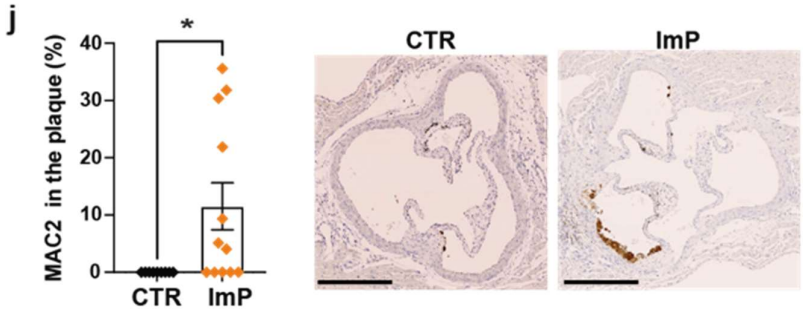
Following the Reviewer's request, we quantified the concentration of ImP in plasma samples from chow-fed *ApoE*$^{-/-}$ mice supplemented with ImP in the drinking water (400µg/mouse/day) for 8 weeks. These results and how they compare to the levels in clinical studies are discussed above in the response to point 10.

13) "scRNA-seq of aortas from 8-weeks treated mice revealed a relative increase in fibroblasts
140 (FBs), endothelial cells (ECs) and immune cells, particularly T and B cells (Fig. 3e), which was confirmed by flow cytometry analysis (Extended Data Fig. 4h)". Macrophage data are needed, especially since they are in Figure 3f.

As requested, we have analyzed total macrophages (CD45+ F4/80+) in the plaque (**New Extended Data Fig. 4g**). Consistent with our results in the scRNAseq analysis, we found similar infiltration of total macrophages in the plaques. However, the scRNAseq analysis reveals that they are transcriptionally different, with increased infiltration of proinflammatory macrophages. This is consistent with our new observation showing increased MAC2^{+} proinflammatory macrophages in the plaques of mice administered ImP compared with control mice (**New Extended Data Fig. 4j**).



New Extended Data 4g. Chow-fed *ApoE*$^{-/-}$ mice were administered ImP or non (CTR) for 8 weeks. Number of CD90^{+} T cells, B220^{+} B cells and F4/80^{+} Macrophages determined by flow cytometry. n=7-10. Individual data and mean ± SEM from at least two pooled independent experiments. Unpaired Student's t test. ns: not significant.



New Extended Data 4j. Chow-fed *ApoE*$^{-/-}$ mice were administered ImP or non (CTR) for 8 weeks. MAC2 staining inside atheroma plaque showing MAC2+ area quantification (left) and representative images (right). n=9-12 Bar size= 500µm. Individual data and mean ± SEM from at least two pooled independent experiments. Unpaired Student's t test. * $p < 0.05$.

14) Please reconcile extended Data Fig 5f of an increased response to Ifg to Fig 3f of strong downregulation of macrophage response to Ifg.

Thanks for noticing the apparent contradiction in these figures that is derived from a mistake in the caption of Figure 3. Fig. 3f is indeed showing the results of a gene set enrichment analysis (GSEA) comparing ImP vs control after 4 weeks of ImP administration, not 8 weeks. Also, we would like to point out that for this GSEA analysis we were not stratifying the different subpopulations of macrophages (MF) but considering the entire cluster. We choose the earlier time point (4 weeks) to better understand the transcriptional effect on cells from aorta that could precede the subsequent proatherogenic phenotype that we found at 8 weeks. At 4 weeks indeed, we detect an incipient development of the atheroma plaque without concomitant findings of systemic inflammation (**Extended Data Fig. 4 e-h**), which were found at 8 weeks (**Fig. 3b-d**). In contrast, in the **Extended Data Fig. 4f**, we performed an overrepresentation analysis (ORA) of the markers that define different macrophage clusters across both time points (4 and 8 weeks) without stratifying by treatment in order to focus on and explore which genes were defining each subpopulation.

Regarding how these two results reconcile (even if they correspond to different time points), it is important to consider that the objective and type of analysis are different. In Fig. 3f, we are comparing ImP vs control using the entire MF population at 4 weeks, whereas in Extended Fig. 4f there is no such comparison.

Anyhow, a possible explanation could be that there might be signals of IFN-related genes that are downregulated in the ImP-condition coming from the other more abundant MF populations that predominate in the whole MF signature. Indeed, the proinflammatory MF3 subset characterized by the Ifg signature is not the most abundant population and in Fig. 3f we were considering the entire MF cluster. In addition, even though IFN response is detected by the GSEA as significantly downregulated in the ImP-stimulated MFs, not all the genes defined by MSigDB as IFN response are changed in the same direction (**Fig. 7 for the Reviewers**). In fact, there are some genes on the top of the rank

such as IFITM3, a gene highly expressed by MF3, that may also contribute to explain the results in Extended Fig. 4f.

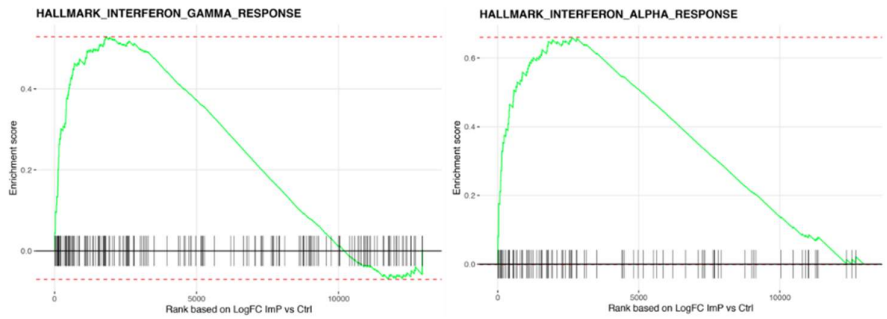


Fig. 7 for the Reviewers. GSEA of IFN-gamma and IFN-alpha response gene sets from MSigDB (https://www.gsea-msigdb.org) comparing ImP (4 weeks) vs Control MFs.

15) "…in vitro studies showed that ImP was able to activate…" Are concentrations used physiologically relevant?

Thanks for raising this point. In our absolute quantification of ImP, we observed physiological mean values of 15nM (range 5.16nM to 27.46nM) and 28nM (range 14.26nM to 57.79nM) in plasma of chow-fed and high cholesterol (HC)-fed *ApoE*$^{-/-}$ mice, respectively (**Fig. 2 for the Reviewers,** see response to point 2). In the case of human healthy volunteers from the PESA cohort with atherosclerosis, the range was 1.26 to 266nM and our cut-off to define a high ImP in plasma was 41nM (**Fig. 2a,e**, and see also response to point 10). In addition, in our validation cohort added in the present manuscript , the IGT cohort, the range was 2 to 899nM (**new Fig. 2a**, see response to point 10) and in previous works we found blood concentration of ImP in humans up to the micromolar range (https://doi.org/10.1016/j.jchf.2023.03.008). Considering this, the treatment of cultured cells with 10µM ImP is thus around 10-fold over the maximal concentrations found in human blood.

Following the Reviewer suggestion to test ImP doses physiologically relevant, we have now performed a dose response in vitro with a range of ImP concentrations from 1nM to 10µM range. We found that ImP was able to induce pS6 activation in BMDMs and MEFs from 10nM (**Fig. 8 for the Reviewers**), indicating its ability to activate mTOR pathway at physiological concentrations.

Of note, many of the results obtained *in vitro* have also been validated upon ImP administration *in vivo*, which results in a physiological range (13.42 to 340nM), as commented in the response to point 10. For instance, pS6 induction by ImP administration in vivo is found in peritoneal MFs (**Fig. 4m**). Moreover, the transcriptomics analysis (scRNA-seq) analysis *in vivo* upon ImP administration also shows activation of MFs and FBs in the aorta (**Fig. 3g**). Thus, we think that the experiments *in vitro* represent the effect of ImP *in vivo*, which could also reach locally higher concentrations than those found in circulation or whose plasmatic concentration

could be attenuated by its association with protein carriers in the serum. Moreover, the results obtained *in vitro* allow us to better understand the potential pathways activated by ImP.

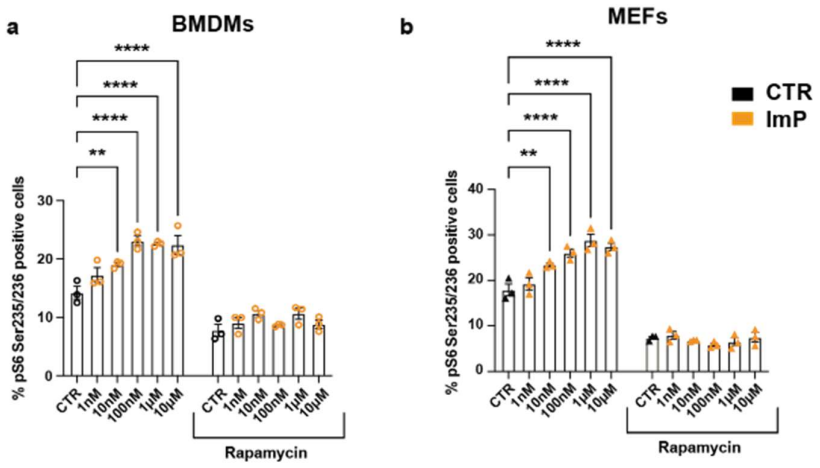


Fig. 8 for the Reviewers. **a,b,** BMDMs and MEFs were treated *in vitro* with the indicated doses of ImP or not (CTR) and co-incubated or not with Rapamycin (mTORC1 inhibitor). Flow cytometry staining for phospho-S6 (pS6) ribosomal protein, showing quantification of percentage of pS6 Ser235/236 positive BMDMs (**a**) and MEFs (**b**). Individual data (each one an independent experiment) and mean ± SEM One-way ANOVA with Tukey post-hoc correction comparing CTR vs ImP. ** p<0.01; **** p < 0.001.

16) Since ImP contains an imidazole ring we hypothesized that ImP could be sensed by ubiquitously expressed imidazoline receptors (IRs) I1R and I2R." This suggests that there could be unappreciated effects of ImP on other organs that could have indirect effects on atherosclerosis. The use of a global inhibitor will not help assess this possibility.

We fully agree with the Reviewer that ImP may be acting not only locally in the artery as revealed by the scRNAseq analysis, but also systemically to promote atherosclerosis. Our results show that ImP in circulation sensed by I1R induces systemic proinflammatory cytokines and immune cell activation (**Figures 3 and 4**), which certainly contribute to the development of atherosclerosis.

Previous publications have correlated ImP with other pathologies such as diabetes (https://doi.org/10.1038/s41467-020-19589-w), hypertension (https://doi.org/10.3390/nu13082706), and even heart failure and all-cause mortality (https://doi.org/10.1016/j.jchf.2023.03.008). We believe that future works will clarify additional effects of ImP and will possibly unveil its involvement in additional pathologies. This reinforces the interest of the new sensing pathway through I1R and its pharmacological targeting that we describe in this manuscript.

Regarding the possibility that the effect of ImP on atherosclerosis is indirect, we would like to stress that the definition of atherosclerosis as a multifactorial disease suggests the involvement of several components, including lipid disbalance and inflammation.

Our results suggest that induction of proinflammatory response by ImP is in the basis of its pathological effect. Following the recommendation from the Reviewer, we have assessed whether ImP administration is capable of inducing some other systemic pathologies that could be confounding in the induction of atherosclerosis, beyond the effect in inflammation.

The induction of type II diabetes is linked to increased plasma glucose resulting from insulin resistance (https://doi.org/10.1007/s11892-022-01453-4). ImP administration did not result in increased glucose concentration in plasma (**Extended Data Fig. 3b)**. Additionally, ImP did not induce significant changes in other parameters related to hypertension or heart failure, such as systolic blood pressure, or renal pathology (albumin and sodium). ImP administration did not affect transaminase levels either (**Fig. 9a-c for the Reviewers),** which, together with the normal cholesterol levels **(Fig. 10 for Reviewers**) indicates that there is no major effect in hepatic function. Moreover, weight gain or triglyceride concentrations in plasma were normal after ImP administration (**Fig. 9 d,e for the Reviewers**).

In any case, in addition to the localized effect observed in the artery by scRNAseq analysis following ImP administration, there is a clear systemic effect in innate and adaptive immunity and we cannot rule out additional systemic effects, as discussed in lines 260ff.

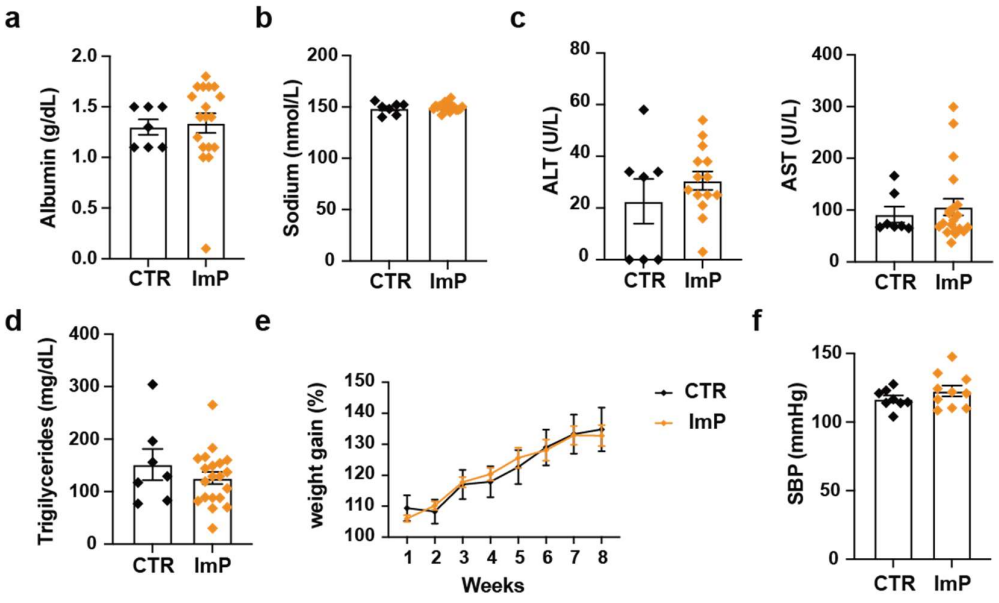


Fig. 9 for the Reviewers. *ApoE*$^{-/-}$ mice were fed chow diet and administered or not (CTR) ImP (400µg/mouse/day) in the drinking water for 8 weeks. **a-d**, Biochemistry parameters quantified in plasma, Albumin (**a**), Sodium (**b**), Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) (**c**), and Triglycerides (**d**). **e**, Weight gain as percentage referred to initial weight. **f**, Systolic blood pressure (SBP) measure by tail-cuff plethysmography. **a-d,f** Individual data and mean ± SEM from two pooled independent experiments. Mann–Whitney U test comparing CTR vs ImP. **e,** Two-way ANOVA test comparing CTR vs ImP.

17) Why are cholesterol levels considerably different in chow fed E-/- mice between Fig 5i and extended data Fig 3a?

Thanks for the insightful comment. The relative difference between groups is similar in both cases, but indeed the cholesterol concentration in WT mice differs between these experiments. These samples were used up so we could not repeat the quantification in the same samples. Thus, we have repeated these experiments to reanalyze the cholesterol concentration. As illustrated in **Fig. 10 for the Reviewers**, and in accordance with Figure 4i and prior literature findings, cholesterol concentration in *ApoE*$^{-/-}$ mice under a chow diet is around 300-400 mg/dL, which can also be slightly affected by the sex and age of the mice (https://doi.org/10.3389/fcvm.2019.00046). We confirm in this new experiment that ImP administration does not affect cholesterol concentration.

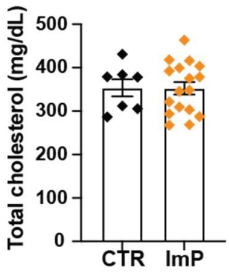
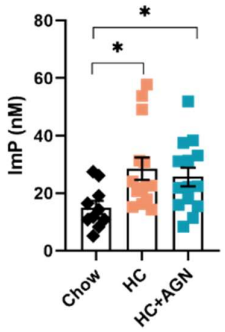


Fig. 10 for the Reviewers. Plasma concentration of total cholesterol in *ApoE*$^{-/-}$ mice feed chow diet and administered or not ImP (400µg/mouse/day) in the drinking water for 8 weeks. Mann–Whitney U test was used to compare CTR vs ImP.

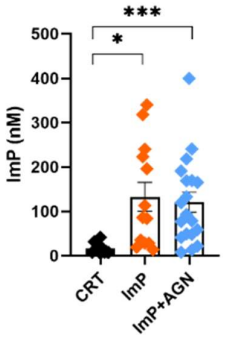
18) In experiments like in Fig 4h, without ImP supplementation, the antagonist decreases atherosclerosis in the high cholesterol diet- but what are the plasma levels of ImP and how do they compare to the values that should have been reported throughout the studies?

We thank the Reviewer for raising this important issue. Through our absolute quantification of ImP, we observed a 2-fold increase in plasma concentration of ImP in mice fed high cholesterol (HC) diet compared to chow-fed mice ($p<0.05$) (**New Extended Data Fig. 8b**). The treatment with AGN192403 did not alter the plasmatic ImP concentration reached when ApoE$^{-/-}$ mice are fed HC diet (**New Extended Data Fig. 8b**).

Similarly, AGN192403 did not affect plasmatic ImP concentration induced by ImP administration in drinking water when the I1R inhibitor was co-administered with ImP in the drinking water of chow-fed *ApoE*$^{-/-}$ mice for 8 weeks (**New Extended data Fig. 7d**), suggesting that the effect of AGN192403 is exerted on I1R without affecting ImP plasma concentration.



New Extended Data Fig. 8b Plasma imidazole propionate (ImP) concentration from *ApoE*$^{-/-}$ mice fed chow or high cholesterol (HC) diets for 8 weeks. At 4 weeks post diet initiation, AGN192403 was administered (AGN) or not in the drinking water to mice fed HC diet until week 8. Individual data and mean ± SEM from two pooled independent experiments. Brown-Forsythe and Welch ANOVA with Dunnett's Post Hoc test ***p < 0.005, *p < 0.05



New Extended Data Fig. 7d. Plasma ImP concentration in chow-fed *ApoE*$^{-/-}$ mice administered or not (CTR) with ImP in the presence of AGN192403 (AGN) or not (ImP) in drinking water for 8 weeks. Individual data and mean ± SEM from two pooled independent experiments. Brown-Forsythe and Welch ANOVA with Dunnett's Post Hoc test *p < 0.05

19) Shigella bacteria go up in % in the HC diet, but the eubacterium in both HCHC and HC diets. In Fig 1f, the ImP relative levels are similarly increased by both diets- does this mean Shigella is more important?

We believe that the ImP increase in plasma likely results from a response to altered microbial ecology illustrated by reduced microbial diversity in mice and previously observed in humans (https://doi.org/10.1038/s41467-020-19589-w and https://doi.org/10.1038/s41587-019-0233-9). ImP is produced by the enzyme UrdA and our data do not allow us to monitor, expression nor enzyme activity thus precluding commenting on the main source of ImP production. This is now discussed in lines 88ff.

20) "ImP and other microbiota-dependent metabolites…." Is it clear what specied of bacteria in the gut is producing ImP?

As discussed above, the resolution of the data does not allow speculation on which bacteria are the main producer of ImP since this would entail metatranscriptomics, enzyme activity measurements as well as isolation of the strains associated with ImP concentration, followed by cloning of UrdA and enzyme characterization. Provided the scope of such investigations, including the limitations in culturing strictly anaerobic bacteria, we consider it to be matter of future studies.

21) "We observed that administration of ImP induced local increase in transcriptional activity in endothelial cells, fibroblasts and macrophages". As noted above, this can be from effects on other organs that secrete factors that induce transcription of various genes- how can this possibility be excluded?

As discussed above in the response to point 16, the administration of ImP indeed induces systemic inflammation. Nevertheless, we have not identified any significant changes in the remaining analyzed parameters associated with renal pathology, hepatic function, hypertension, or metabolic syndrome, suggesting ImP increase has a relatively selective effect in the induction of an inflammatory profile that could underlie some inflammatory or autoimmune diseases beyond atherosclerosis. The fact that ImP administration is having a local effect as detected by scRNAseq indeed does not preclude that this is the result of a systemic impact of the metabolite, and indeed we found systemic effects in innate and adaptive immunity. This is discussed in lines 260ff.

22) Does the receptor do anything good? In other words, is there a potential downside to blocking it?

Thanks for this interesting comment. Our data suggest that ImP could induce a systemic proinflammatory profile. The induction of a local inflammatory profile could be beneficial in the context of cancer. Several publications have focused on analyzing the role of Nischarin (I1R) in cancer cells (https://doi.org/10.1002/ijc.32690; https://doi.org/10.2147/ott.s228317). Reduced expression of Nischarin are associated with increases migration and invasion of surrounding tissues by cancer cells. Testing whether agonist action on I1R could affect cancer growth is an interesting separate project beyond this manuscript.

Referee #2 *(Remarks to the Author):*

Mastrangelo et al. aimed to study the impact of imidazole proportionate (ImP), a microbiota-dependent metabolite on atherosclerosis. This metabolite has previously been identified as a key factor involved in insulin resistance and was associated with heart failure and all-cause mortality. Herein, the authors showed that this metabolite exerts a pro-atherogenic role. This was related to an increase in systemic and local atherosclerotic plaque inflammation, without impacting plasma cholesterol levels. This effect was related to the activation of imidazoline receptor (I1R).

1. While the demonstration of the pro-atherogenic role of ImP is important, the mechanistic insights are limited, which dampens the enthusiasm for this paper for Nature. The results are mainly descriptive showing an increase in inflammation without giving a clear demonstration of the underpinning mechanisms from activation of I1R by ImP to inflammation and atherosclerosis.

We agree with the Reviewer that it is interesting to further analyze the mechanisms by which ImP is inducing atherosclerosis as well as the specific cell types that could play a crucial role in this process. To delve deeper into this question, we have conducted various *in vitro* analyses, including RNAseq of bone marrow derived macrophages (BMDMs), mouse embryonic fibroblasts (MEFs) and mouse aortic endothelial cells (MAECs) (**New Fig. 3h and Extended Data. Fig. 5j**) to complement the data obtained upon *in vivo* ImP administration by scRNAseq (**Fig. 3g**). To further investigate signaling pathways triggered by ImP in these cellular targets and connecting them with the induction of an inflammatory profile that results in atherosclerosis, we have performed proteomic-phosphoproteomic analyses upon stimulation with ImP in BMDMs and MEFs, selected as the main targets of ImP following the RNAseq analysis (**New Fig. 3i and New Extended Data. Fig. 5k-m**).

The RNAseq analysis revealed that the expression of I1R (encoded by *Nisch*) is higher in BMDMs and MEFs compared with MAECs (**Fig. 11 for the Reviewers**). Moreover, RNAseq analysis showed rapid induction of proinflammatory genes in BMDMs and MEFs, but not MAECs, following ImP treatment (**New Fig. 3h and New Extended Data Fig 5j)**, suggesting that MFs and FBs are the main targets of ImP.

To assess potential signaling pathways underlying the induction of this inflammatory profile, we conducted a proteomics and phosphoproteomics analysis in MEFs and BMDMs. This study revealed increased phosphorylation in peptides corresponding to proteins related to activation of the mTOR pathway (**New Fig. 3i and New Extended Data. Fig. 5k**). Since ImP administration induces pS6 and TNFα production and is inhibited by rapamycin (**Extended Data Fig. 5l,m**) these results suggest that ImP induces an inflammatory profile through activation of the mTOR pathway. This is consistent with previous studies describing increased activation of this pathway in the liver of patients with type 2 diabetes mellitus (https://doi.org/10.1016/j.cell.2018.09.055). Of note, our results also show that AGN192403 blockade of I1R prevents the activation of this pathway, as demonstrated by our phosphoproteomics studies (**new Fig. 3i**), and results

in pS6 and TNFα production (**Extended Data Fig. 6a,b**). Moreover, we have now analyzed silencing of I1R as an additional approach to test the specific action of ImP through I1R (**see response to point 6, below**).

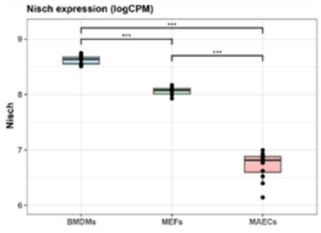
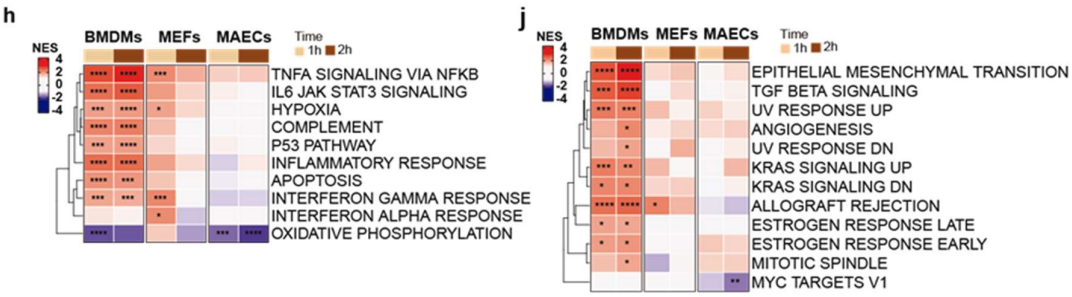
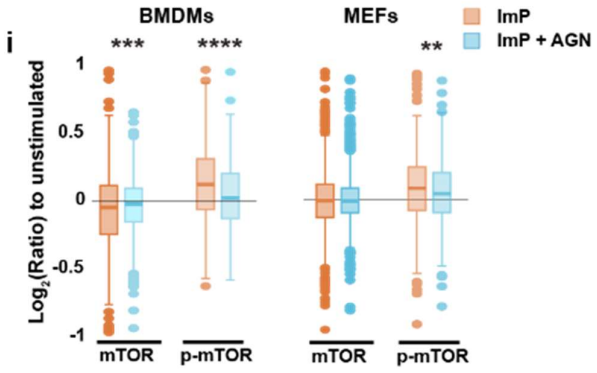


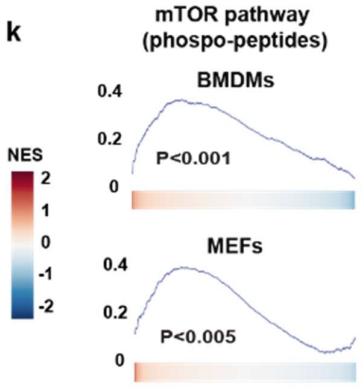
Fig. 11 for the Reviewers. Normalized expression values of *Nisch* in BMDMs, MEFs, and MAECs. Walt test using the R DESeq2 package (https://doi.org/10.1186/s13059-014-0550-8). *** p<0.005



New Fig. 3h.(left) and New Extended Data Fig 5j (right). Heat map of gene-set enrichment analysis from RNAseq analysis comparing ImP stimulation after 1h and 2h with unstimulated within each cell type. Nominal p-values calculated using a 1000-permutation test. * p<0.05; *** p<0.005; **** p<0.001.



New Fig. 3i, Comparison of the global abundance of peptides (mTOR) and phosphopeptides (p-mTOR) from the mTOR pathway in BMDMs (left) or MEFs (right) upon treatment with ImP for 3h in the presence or not of AGN192403. Peptides and phosphopeptides are quantified as Log2 ratio and referred to unstimulated condition. Wilcoxon Signed Rank comparing ImP vs ImP + AGN. *** p<0.005; **** p<0.001.



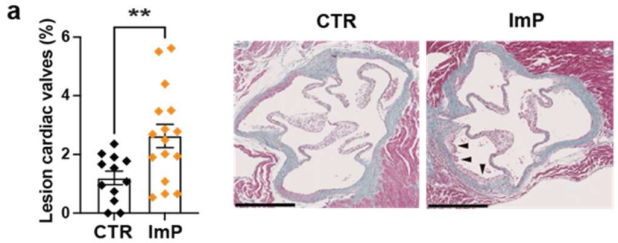
New Extended Data Fig. 5k. Gene Set Enrichment Analysis comparing phosphopeptides and peptides related to the mTOR pathway after ImP stimulation in BMDMs (top) and MEFs (down). Nominal p-values are calculated using a 1000-permutation test.

I have other major concerns as follows:

2. -atherosclerosis-related parameters are not well investigated, the authors should examine other atherosclerotic sites such as the aortic sinus, which is classically investigated in atherosclerosis-related studies. Also, they should examine inflammatory cell accumulation in the aortic sinus plaques

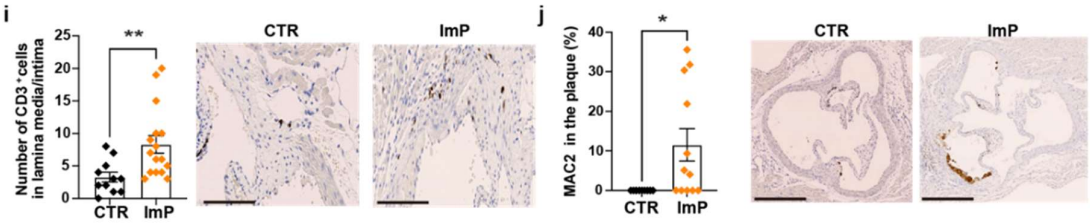
Following the Reviewer's recommendation, we have complemented the analysis of atherosclerotic plaques by examining their extent as well as their composition in aortic sinuses. Similar to the aortic arch and aorta, we found that ImP is capable of inducing atherosclerotic plaques (**New Extended Data Fig. 3a),** along with increased infiltration of T cells in the medial/intimal layer and foam macrophages in the plaque (**New Extended Data Fig. 4i,j)**. We did not observe induction of necrotic core, caspase 3 positive staining or changes in vessel structure following ImP administration, suggesting that ImP-induced plaques are in an early stage of development (**Fig. 3 for the Reviewers).**

In addition, we analysed the effect of treatment with AGN192403 in the changes in the composition and stability of the plaque upon HCD diet-induced atherosclerosis as explained below in the response to point 5.



New Extended Data Fig. 3a. *ApoE*$^{-/-}$ mice were administered ImP or not (CTR) for 8 weeks. Masson's trichrome staining of the aortic valves performed under the indicated

treatments. Left: Quantification of atherosclerotic lesions as percentage of total valve area. Right: Representative images. Arrowheads indicate lesions. n=12-16. Individual data and mean ± SEM from at least two pooled independent experiments. Unpaired Student's t test. ** p < 0.01.



New Extended Data Fig. 4i,j, *ApoE*$^{-/-}$ mice were administered ImP or not (CTR) for 8 weeks. **i**, Staining of CD3+ cells in intima/media layer in aortic valves showing quantification (left) and representative images (right) of infiltrated T cells after 8 weeks of ImP administration. n=11-16. Bar size = 500µm. **j**, MAC2 staining inside atheroma plaque showing MAC2+ area quantification (left) and representative images (right). n=9-12, Bar size = 500µm. Individual data and mean ± SEM from at least two pooled independent experiments. Unpaired Student's t test. * p < 0.05; ** p < 0.01.

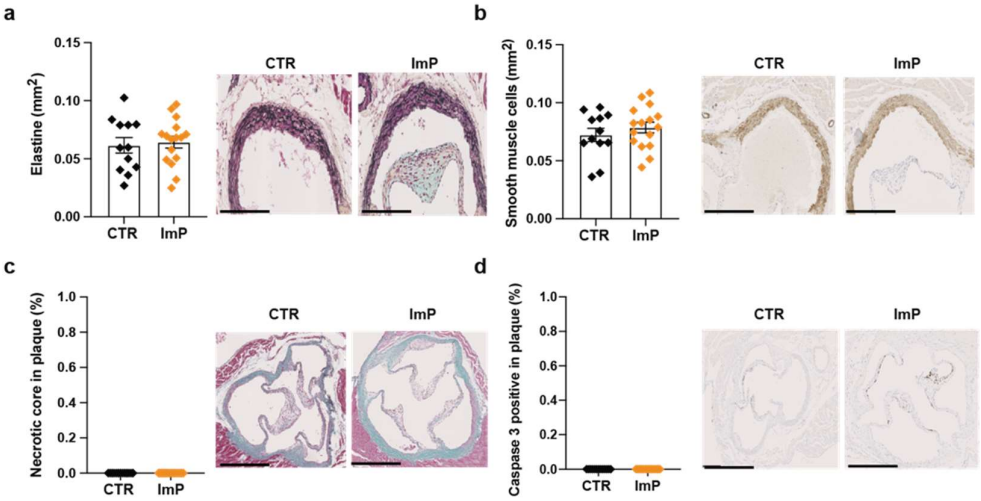


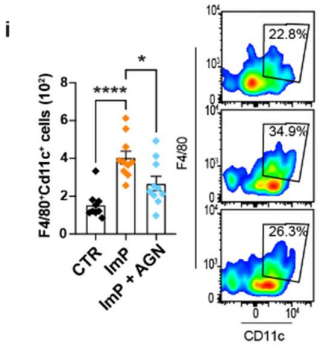
Fig. 3. for the Reviewers. *ApoE*$^{-/-}$ mice were fed a chow diet for 8 weeks and treated or not (CTR) with ImP in the drinking water. **a**, Integrity of elastin fibers, quantified as area of elastin with Movat's pentachrome staining. Black areas were used to quantified Elastin fibers, showing quantification (left) and representative images (right), n=12-16. **b**, Integrity of smooth muscle cells (SMC) quantified as area of SMC using SMA antibody, showing quantification (left) and representative images (right), n=12-16. **c**, Percentage of necrotic core area inside the total area plaque, quantified as non-Masson's trichrome staining areas in cardiac plaque showing quantification (left) and representative images (right), n=13-17. **d,** Quantification of Caspase 3 positive stain in cardiac valves lesion showing quantification (left) and representative images (right), n=13-17. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction.

3. -the authors should deeply investigate the inflammatory profile of macrophages within the plaques not only facs staining of F4/80+ CD86+ as "inflammatory macrophages"

We have further characterized the contribution of inflammation and immunity in the context of ImP-induced atherosclerosis. As commented above in our response to point 2, we further dissected plaque composition by the combined analysis of the aorta by flow cytometry and the histological analysis of aortic valves in chow-fed *ApoE*$^{-/-}$ mice administered or not (CTR) ImP (400µg/mouse/day) in the drinking water for 8 weeks. The ImP group was further treated (ImP+ AGN) or not with AGN192403 in the last 4 weeks to prevent progression of ImP-induced atherosclerosis. Through flow cytometry, we identified an increased infiltration of F4/80+CD11c+ cells in the aorta (**New Extended Data Fig. 7i**), previously linked to atherosclerotic plaque development in *ApoE*$^{-/-}$ mice (https://doi.org/10.1038/s41467-021-27862-9). These macrophages are broadly associated with elevated production of proinflammatory cytokines (https://doi.org/10.1189/jlb.1106680). Their role is complementary to the increased presence of F4/80+CD86+ cells (**Fig. 4g**), which provide co-stimulatory signals inducing heightened activation of T cells (https://doi.org/10.1002/iid3.740).

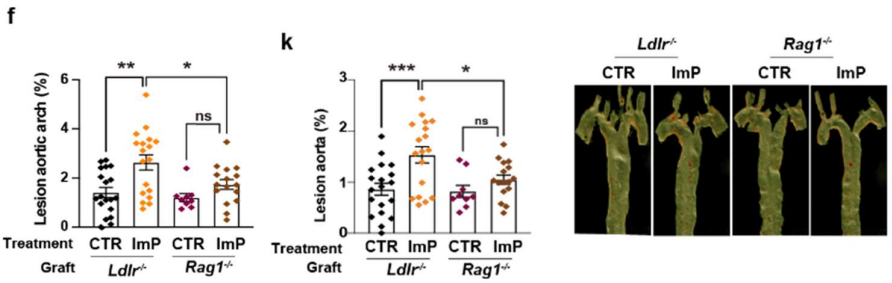
In the flow cytometry analysis, we did not find a difference in macrophage numbers (**Extended Data Fig. 4g** consistent with our results in scRNAseq showing similar macrophage numbers in the aorta (**Fig. 3e**). Nevertheless, we observed a higher number of infiltrated T cells in the medial/intimal layer of the vascular wall (**New Extended Data 4i**), indicative of increased inflammation in it. Linked to this heightened T cell infiltration in the plaque, we observed an increased systemic polarization towards Th17 and Th1 cells in blood (**Fig. 3c,d**).

To further investigate the key role of T cells, we used *Rag1*$^{-/-}$ mice, deficient in mature T and B cells. Bone marrow transplantation was conducted in *Ldlr*$^{-/-}$ mice, which were treated or not with ImP in the drinking water. *Ldlr*$^{-/-}$ mice grafted with *Rag1*$^{-/-}$ bone marrow did not develop atheroma plaques in response to ImP (**New Fig. 3f and Extended Data Fig. 3e**), indicating that adaptive immunity was needed for the induction of atherosclerosis in response to ImP.



New Extended Data Fig. 7i. ImP was administered (ImP) or not (CTR) in the presence of AGN192403 (AGN) or not in drinking water to *ApoE*$^{-/-}$ mice fed chow diet for 8 weeks, followed by sacrifice and analysis. Number of F4/80^{+} Cd11c^{+} cells in the aorta by flow cytometry. n=10. Arithmetic mean ± SEM and individual data from at least two pooled

independent experiments. One-way ANOVA with Tukey post-hoc correction. * p < 0.05; **** p < 0.001.

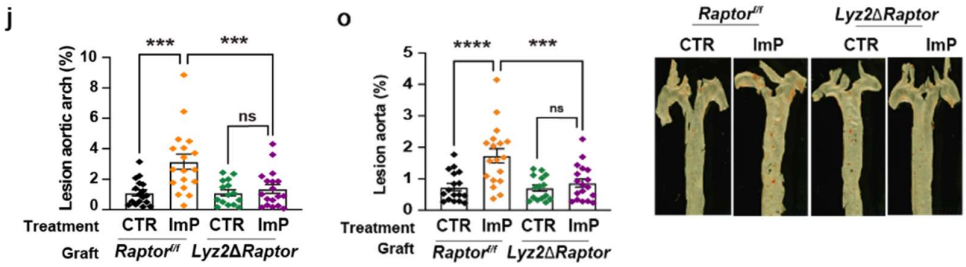


New Fig. 3f and Extended Data Fig. 4k. ImP was administered (ImP) or not (CTR) in drinking water for 12 weeks to *Ldlr*$^{-/-}$ mice grafted with BM from *Rag1*$^{-/-}$ or *Ldlr*$^{-/-}$ mice. Quantification of atherosclerotic lesion by oil red O en face staining of the aorta showing quantification in the aortic arch (**f**), in whole aorta (**k**, left) and representative images (**k**, right). One-way ANOVA with Tukey post-hoc correction. n=9-19. *p<0.05; **p<0.01; ***p<0.005.

4. - *How do they connect the results regarding pS6 (as a surrogate of mTOR activation) with the observed phenotype? Moreover, why they analyzed pS6 induction only in peritoneal macrophages and not locally within the plaques? What would be the effects of the mTOR inhibitor treatment on the phenotype in vivo?*

Thanks for this interesting question. Based on our new results on the RNAseq and proteomics/phosphoproteomics analysis in response to ImP, we hypothesized that ImP-triggered mTOR activation could be crucial for induction of a proinflammatory profile that contributes to atherosclerosis progression. Regarding the effects of a systemic mTOR inhibitor treatment on the phenotype, as suggested by the Reviewer, it is well established that rapamycin (mTOR inhibitor) prevents atherosclerosis development (https://doi.org/10.1034/j.1600-6143.2003.00094.x, https://doi.org/10.1016/j.atherosclerosis.2003.09.003, https://doi.org/10.1097/01.fjc.0000177985.14305.15, https://doi.org/10.1590/S0100-879X2009005000036, https://doi.org/10.1111/j.1476-5381.2008.00080.x, https://doi.org/10.1186/s12929-016-0274-z, https://doi.org/10.1016/j.intimp.2017.02.026, https://doi.org/10.1074/jbc.M117.779447).

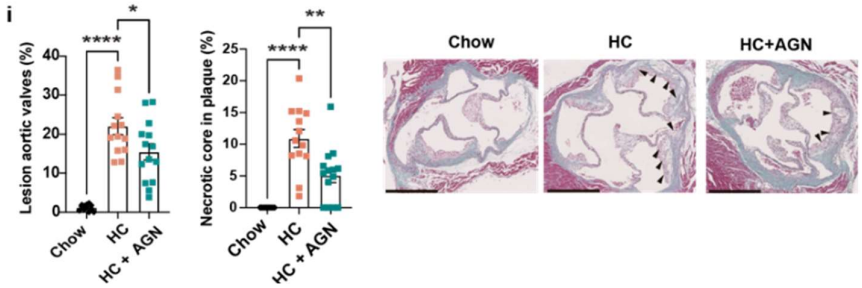
Thus, to get further insights and address the specific contribution of mTOR in myeloid cells to the development of ImP-induced atherosclerosis, we generated *Lyz2*Cre x *Raptor*$^{f/f}$ (*Lyz2*Δ*Raptor*) mice with impaired mTOR activation in the myeloid compartment. We transplanted BM from *Lyz2*Δ*Raptor* or control *Raptor*$^{f/f}$ mice to *Ldlr*$^{-/-}$ recipients that were treated (ImP) or not (Ctrl) with ImP (400µg/mouse/day) in the drinking water. The results showed that Raptor deficiency in the myeloid compartment completely prevented the development of atherosclerosis by ImP (**New Fig. 3j and Extended Data Fig. 5o**), demonstrating the key role of mTOR in myeloid cells in this process.



New Fig. 3j and Extended Data Fig. 5o. ImP was administered (ImP) or not (CTR) to *Ldlr*$^{-/-}$ mice grafted with BM from control *Raptor*$^{f/f}$ or *Lyz2ΔRaptor* and fed chow diet for 12 weeks. Quantification of atherosclerotic lesion by oil red O en face staining of the aorta showing quantification in aortic arch **(j)** and in whole aorta and representative images **(o).** n=15-18. Individual data and mean ± SEM of at least two pooled independent experiments. Unpaired Student's t test comparing CTR and ImP; One-way ANOVA with Tukey post-hoc correction. *** $p < 0.005$; **** $p < 0.001$.

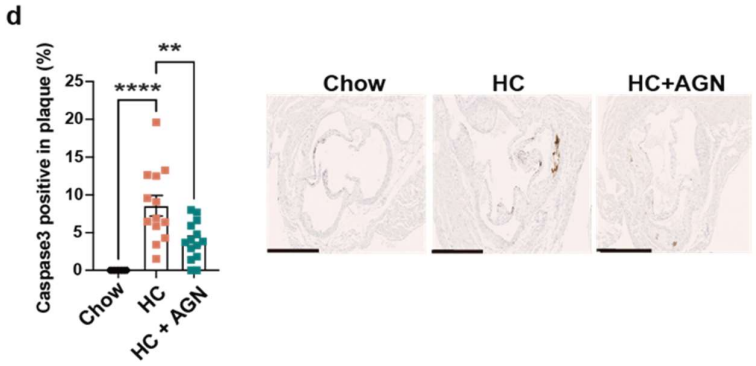
-5. concerning the effects of I1R blockage on atherosclerosis progression, the authors contented to analyse systemic inflammation without investigating inflammation within the plaques.

To further investigate the effect of I1R blockade with AGN192403 on the plaque status, we conducted histological analysis of plaque composition in the aortic valves in *ApoE*$^{-/-}$ mice fed or not (Chow) HC diet for 8 weeks and treated (HC+AGN) or not (HC) with AGN192403 in the last 4 weeks of diet. Similar to the findings by oil red O staining in the aorta and aortic arch, AGN192403 treatment in the last 4 weeks of HC diet was able to reduce the development of atherosclerotic plaque in the aortic valves (**New Fig. 4i**). Additionally, AGN192403 administration resulted in lower expression of caspase 3, along with a decrease in the necrosis area within the atherosclerotic plaque (**New Fig. 4i and new Extended Data Fig. 8d**), indicating diminished cell death, which is associated to reduced plaque complexity in mice (https://doi.org/10.1038/s41467-023-36614-w; https://doi.org/10.1161/atvbaha.122.318177).



New Fig. 4i, *ApoE*$^{-/-}$ mice were fed chow diet or high cholesterol (HC) diet for 8 weeks. At 4 weeks post diet initiation, AGN192403 was administered (AGN) or not in the drinking water to mice fed HC diet until week 8, followed by sacrifice and analysis. **i**, Masson's trichrome staining of aortic valves. Quantification of lesion in aortic valves (left), necrotic core area (middle) and representative images (right). Arrowheads

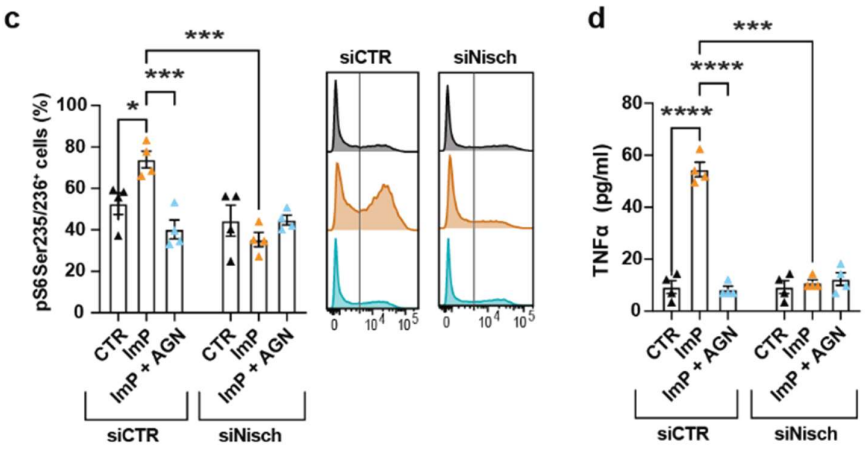
indicate necrotic areas. Bar size= 500µm. n=12-14. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.001$.



New Extended Data Fig. 8d, *ApoE*$^{-/-}$ mice were fed chow diet or high cholesterol (HC) diet for 8 weeks. At 4 weeks post diet initiation, AGN192403 was administered (AGN) or not in the drinking water to mice fed HC diet until week 8, followed by sacrifice and analysis. **d,** Caspase 3 staining of aortic valves. Quantification of caspase 3+ (left) and representative images of Caspase 3 staining (right) Bar size= 500µm. n=12-14. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. ** $p < 0.01$; **** $p < 0.001$.

6. -the authors should demonstrate the specificity of I1R antagonist. The use of I1R KO in this context would be valuable

Several publications have described the specificity of AGN192403 as a selective inhibitor of I1R over other imidazoline receptors or the secondary action of some of these on α2-adrenergic receptors (https://doi.org/10.1093/genetics/85.4.713; https://doi.org/10.1016/j.brainres.2020.146695). I1R KO mice were not available in the timeframe conceded by the Editor to address the comments. Thus, in order to address this comment, we used a silencing strategy, using Nisch siRNA (sc-61202, https://doi.org/10.4103/1673-5374.217348) vs control siRNA (sc-36869) transfected in MEFs to validate that ImP cellular effects (e.g. pS6 induction and TNFα production) were mediated by I1R and that AGN192403 is specific for I1R and has no effect in I1R-silenced setting. Silencing of I1R by Nisch siRNA in MEFs blocked ImP-induced pS6 and TNFα production, revealing that ImP signals selectively through I1R for these inflammatory functions. Of note, AGN192403 prevented ImP-induced pS6 or TNFα in control siRNA treated cells but had no effect in I1R silenced cells, consistent with the selective effect of this inhibitor on the ImP/I1R axis (**New Extended Data Fig. 6c,d**).



New Extended Data Fig. 6 c,d. MEFs were transfected with I1R siRNA (siNisch) or control siRNA (siCTR) and stimulated with ImP or not (CTR) and co-incubated or not with AGN192403 (AGN) as indicated. **c**, Flow cytometry staining for phospho-S6 (pS6) ribosomal protein, showing quantification of percentage of pS6 Ser235/236 phosphate positive cells (left) and representative histograms (right) corresponding to each treatment indicated and matched by color. **d**, TNFα production in 24h culture supernatant by ELISA. **c,d**, Arithmetic mean ± SEM and individual data from n=4 pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$.

7. -The connection between microbiota and ImP is missing in the experimental studies. The assessment of the specific bacteria involved in ImP-mediated proatherogenic effects would have been appreciable.

We agree with the referee on the relevance of knowing which species of bacteria in the gut are producing ImP. However, this is not the main objective of our work, rather the downstream effects of ImP in atherosclerosis and exploration of underlying disease mechanisms. We believe it is an important topic of research that deserves further exploration in future work. Furthermore, as stated in the response to Reviewer 3, point 9, this is a huge undertaking to provide sufficient resolution in the experiments and taken together we consider it to be outside the scope of the current study.

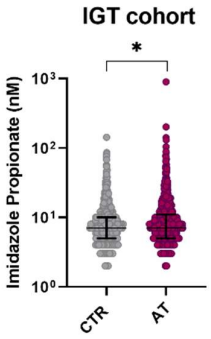
Referee #3 *(Remarks to the Author):*

Mastrangelo and colleagues combine human and mechanistic mouse studies to show that Imidazole Propionate (ImP) contributes to atherosclerosis and systemic inflammation without impacting cholesterol. Study also suggest that ImP could be used in combination with other risk factors to diagnose atherosclerosis at early stages and provide evidence suggesting Imidazoline receptor 1 as the potential target of ImP. While the studies provide novel epidemiological and molecular insights, there are several issues that need to be addressed.

Major:
1. Inclusion of a validation cohort would strengthen the manuscript.

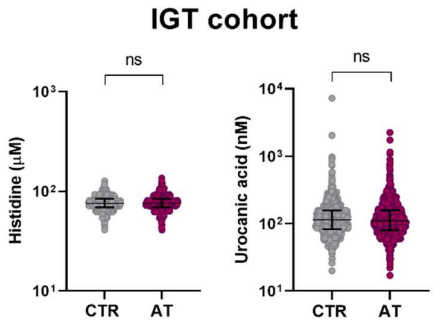
We would like to acknowledge the Reviewer for this insightful comment. To address this request, we carried out the quantification of ImP and its related metabolites in the Swedish IGT cohort (https://doi.org/10.1016/j.ahj.2024.01.011). Similar to the PESA cohort, the IGT cohort enrolled volunteers from the general healthy population (men and women from 50 to 64 years) performing detailed imaging and functional analyses of the cardiovascular system. This has enabled detecting subclinical stages of the disease (https://doi.org/10.1016/j.ahj.2024.01.011) . Consistent with our approach, subclinical atherosclerosis in IGT cohort was assessed by vascular ultrasonography of carotid arteries, a well-validated non-invasive technique for detecting, quantifying and staging subclinical atherosclerosis (https://doi.org/10.1038/ncpneuro0324).

Plasma samples were collected from fasted subjects and analyzed by a targeted metabolomics experiment, allowing accurate quantification of ImP and its related metabolites (urocanic acid and histidine). As depicted below, we successfully validated our findings in this independent cohort: 1. ImP plasma concentration is selectively increased in subjects with subclinical atherosclerosis when compared to controls; 2. histidine and urocanic acid concentrations did not show any significant difference between the two groups. These results have been included into the **New Fig. 2a** of the manuscript, and additional details, including study population characteristics and targeted metabolomics method, are provided in the **New Extended Data Table 2** and the Methods section.



New Fig. 2a: Plasma concentration of imidazole propionate (ImP) in healthy subjects (CTR, n=838) and subjects with subclinical atherosclerosis (AT, n=1006) from the IGT

cohort. Arithmetic mean ± SEM of each group is shown. Mann–Whitney U test. *p<0.005.



New Extended Data Fig. 2a: Plasma concentration of metabolites of the imidazole propionate (ImP) biosynthetic pathway, including histidine (left) and urocanic acid (right), in healthy subjects (CTR, n=838) and subjects with subclinical atherosclerosis (AT, n=1006) from the IGT cohort. Arithmetic mean ± SEM of each group is shown. Mann–Whitney U test. ns: not significant.

2. Why does high cholesterol increases ImP in mice? This is not an expected finding as cholesterol is not a substrate for ImP production. If it is known how chol changes ImP please provide a reference. Is inflammation of the gut/availability of electron acceptors such as O2/NO3 involved in in ImP increase in response to HC diet? Is the increased cause by HC causes similar increases as detected in humans? Or when mice supplemented with ImP in drinking water?

We agree that cholesterol is not a substrate of UrdA. Previous data have demonstrated that an unhealthy diet (assessed by the alternate Healthy Eating Index (aHEI), Dietary Approaches to Stop Hypertension (DASH) score, Dietary Diversity Score (DDS) and Mediterranean diet score), but not histidine intake, was associated with increased ImP concentration in plasma. This included a positive association between ImP and saturated fatty acids, while we did not examine dietary cholesterol in that study. Accordingly, these findings suggest that rather than being affected by histidine as a substrate, increased ImP production may be, at least in part, the result of an unhealthy diet changing the microbiota composition and diversity. Cholesterol enriched diets shift the microbiome composition and it is not unlikely that the diet also shift the environment in the gut leading to altered expression and activity of this enzyme resulting in increased production of ImP, potentially by driving altered response to oxygen and nitrate (https://doi.org/10.1126/science.aba3683). While this is a very interesting topic, it is not the main focus of this manuscript.

3. Looks like the team has capabilities to measure ImP concentrations; mouse ImP data should be provided in concentrations so it is clear what levels the mice are exposed to in the different treatments and how this compares to conc. measured in humans?

We would like to thank the Reviewer for this comment. Following the Reviewer's request, we quantified the concentration of ImP in plasma samples from *ApoE*$^{-/-}$ mice following different treatments, as shown in **Fig. 12 for the Reviewers**: **a**, supplementation of ImP in drinking water (400µg/mouse/day) to chow-fed mice for 8 weeks; **b**, feeding a high cholesterol (HC) diet for 8 weeks. In both models, we observed a statistically significant increase of ImP plasmatic concentration when compared to chow-fed *ApoE*$^{-/-}$ mice ($p<0.05$) (**a,b**). Specifically, we noted an 8-fold increase for mice supplemented ImP in the drinking water and a 2-fold increase for mice fed a HC diet. When comparing these results with the ImP concentration quantified in humans from the PESA cohort (**c**), we observed similar mean concentration between mice and humans, particularly in the case of the HC diet, with a 1.6-fold increase in humans compared to chow-fed mice. The IGT cohort depicted in response to point 1 also showed a similar range (**New Fig. 2a**). Interestingly, by supplementing ImP in the drinking water, ImP concentration was 5-fold higher in chow-fed mice than in humans from the PESA cohort. Of note, the highest concentration of plasmatic ImP in mice supplemented with ImP and in humans from the PESA cohort with subclinical atherosclerosis are comparable (340nM in mice, 266nM in the PESA cohort), and this range of ImP concentration was even higher (up to 899nM) in the measurements performed in Sweden in the IGT cohort (see response to point 1), suggesting that the concentration reached by ImP upon its administration in the drinking water could be similar to those of humans with early atherosclerosis.

These new data on ImP absolute concentrations are now included in the manuscript (**new Fig. 2a; Fig. 2b; New Extended Data Fig. 7d; and New Extended data Fig. 8b**).

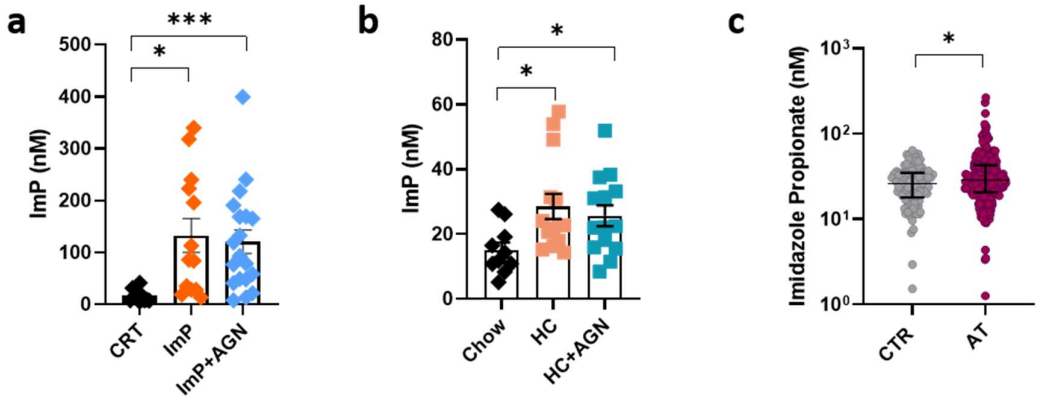


Fig. 12 for the Reviewers. a, Plasma ImP from *ApoE*$^{-/-}$ mice fed chow diet and supplemented with ImP in the drinking water (400µg/mouse/day) for 8 weeks. **b,** Plasma imidazole propionate (ImP) from *ApoE*$^{-/-}$ mice fed chow or high cholesterol (HC) diets for 8 weeks. **a,b** Individual data and mean±SEM from two pooled independent experiments. Welch test **p < 0.001. **c,** Plasma ImP in healthy subjects (CTR, n=105) and subjects with subclinical atherosclerosis (AT, n=295) from the PESA cohort. Median and interquartile range of each group is shown. Mann–Whitney U test. *p<0.05.

4. Fig 2. Is FDR correction applied to correl analysis?

Following the Reviewer's suggestion, we performed the Benjamini-Hochberg FDR correction for the correlation analysis showed in Fig. 2. As a result of this analysis, some variables including familial history of hypertension, the relative abundance of two bacterial families (i.e. *Actinomyces* and *Megasphaera)*, and age, were no longer found to be significantly associated with plasma concentration of ImP. Consequently, the significant association between these variables and ImP have been excluded from **Fig. 2d** of the manuscript.

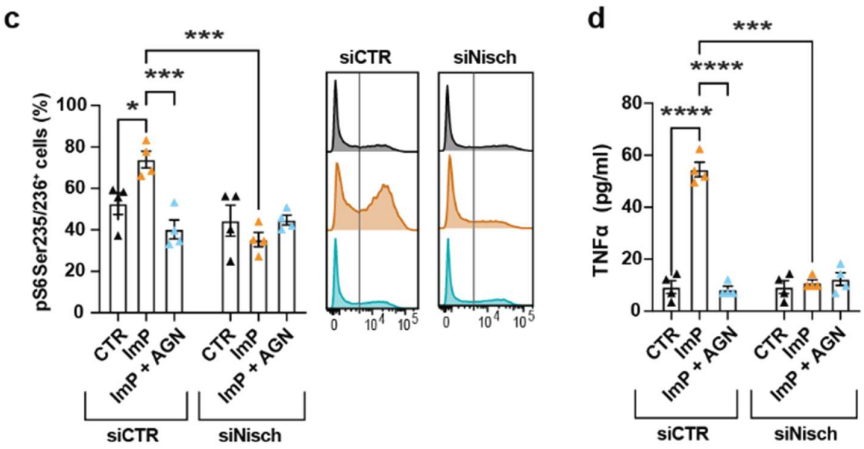
Table. Results of the Benjamini-Hochberg FDR correction for the correlation analysis between imidazole propionate and different atherosclerosis pathophysiological features, diet and microbiota

Variable	**p-value**	**Adjusted p-value**
ALT	0	0
BMI	0	0
Sex	0	0
Acidaminococcus	0	0
ECO3D FEML	0.001	0.006
SUVMAX BM	0.001	0.006
Fasting Glucose	0.001	0.006
Creatinine	0.002	0.008727
Visceral fat	0.002	0.008727
Vegeterian (Pattern 2)	0.002	0.008727
ECO2D FEM L	0.003	0.011077
Hypercolesterolemiaf	0.003	0.011077
Treatment dyslipidemia	0.006	0.018
Hemoglobin	0.006	0.018
Erysipelotrichaceae	0.006	0.018
Uric acid	0.009	0.022737
GGT	0.009	0.022737
ECO2D FEM R	0.009	0.022737
HDL-C	0.01	0.022857
Hs-C reactiveprotein	0.01	0.022857
DBP	0.013	0.02713
SBP	0.013	0.02713
Coriobacteriaceae	0.016	0.032
Triglyceride	0.018	0.03456
Obesity	0.021	0.038769
Veillonella	0.022	0.039111
Total Calcium Score	0.025	0.041379
Mediterrean (Pattern 1)	0.025	0.041379
Dyslipidemia	0.027	0.0432
ECO3D FEM R	0.032	0.049548

Hypertension_F	0.035	0.050909
Megasphaera	0.035	0.050909
Actinomyces	0.037	0.052235
Age	0.047	0.064457
AST	0.051	0.068
Glycohemoglobin	0.135	0.175135
HTN	0.142	0.179368
Treatment HTN	0.178	0.219077
Active smoking	0.241	0.2892
Escherichia/Shigella	0.253	0.296195
Dialister	0.369	0.421714
Streptococcus	0.482	0.538047
Social (Pattern 5)	0.599	0.653455
Western (Pattern 4)	0.682	0.727467
Breakfast (Pattern 3)	0.791	0.825391
Albumin/Creatinine	0.862	0.88034
Pasteurellaceae	0.882	0.882

5. While the results with AGN192403 support the notion that ImP binds to IR1, this is not formally tested. Knockdown or cell specific (?) deletion IR1 showing reversion of phenotypes would strengthen arguments.

Several publications have described the specificity of AGN192403 as a selective inhibitor of I1R over other imidazoline receptors or the secondary action of some of these on α2-adrenergic receptors (https://doi.org/10.1093/genetics/85.4.713; https://doi.org/10.1016/j.brainres.2020.146695). I1R KO mice were not available in the timeframe conceded by the Editor to address the comments. Thus, in order to address this comment, we used a silencing strategy, using Nisch siRNA (sc-61202, https://doi.org/10.4103/1673-5374.217348) vs control siRNA (sc-36869) transfected in MEFs to validate that ImP cellular effects (e.g. pS6 induction and TNFα production) were mediated by I1R and that AGN192403 is specific for I1R and has no effect in I1R-silenced setting. Silencing of I1R by Nisch siRNA in MEFs blocked ImP-induced pS6 and TNFα production, revealing that ImP signals selectively through I1R for these inflammatory functions. Of note, AGN192403 prevented ImP-induced pS6 or TNFα in control siRNA treated cells but had no effect in I1R silenced cells, consistent with the selective effect of this inhibitor on the ImP/I1R axis (**New Extended Data Fig. 6c,d**).



New Extended Data Fig. 6 c,d. MEFs were transfected with I1R siRNA (siNisch) or control siRNA (siCTR) and stimulated with ImP or not (CTR) and co-incubated or not with AGN192403 as indicated. **c**, Flow cytometry staining for phospho-S6 (pS6) ribosomal protein, showing quantification of percentage of pS6 Ser235/236 phosphate positive cells (left) and representative histograms (right) corresponding to each treatment indicated and matched by color. **d**, TNFα production in 24h culture supernatant by ELISA. **c,d**, Arithmetic mean ± SEM and individual data from n=4 pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$.

6. An novel result presented here is that ImP exacerbates athero development without changes in cholesterol in mice that have relatively low cholesterol levels (Extended Data Fig. 3), however all the results presented are either in ApoE and Ldlr ko mice. Current paradigm is that hypercholesterolemic background is necessary to induce athero in mice. Is ImP sufficient to exacerbate inflammation/exacerbate athero in wt mice?

This is a very interesting experiment proposed by the Reviewer, since ImP induced atherosclerosis in chow-fed *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice without altering lipid levels could suggest that this atherosclerosis-prone background was not needed. As suggested, we treated C57BL/6J mice with 800µg/mouse/day of ImP for 23 weeks. After 23 weeks, we did not observe atherosclerotic plaques in the mice treated with ImP compared to the control group (**Fig. 13 for the Reviewers**). This result indicates that although the effect of ImP is independent of changes in cholesterol concentration, a proatherogenic mouse background with higher concentration of cholesterol may be necessary. Indeed in both experimental models, 200-400 mg/dL in *Ldlr*$^{-/-}$ mice (https://doi.org/10.1172/jci116663) and 400-600 mg/dL in *ApoE*$^{-/-}$ mice (https://doi.org/10.3389/fcvm.2019.00046), cholesterol concentration was higher than in C57BL/6J mice (i.e. between 80-100 mg/dL, https://doi.org/10.1161/01.atv.14.1.133) in steady-state conditions when fed a chow diet.

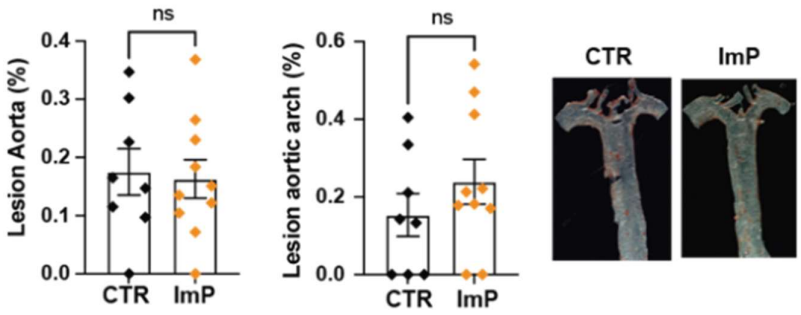
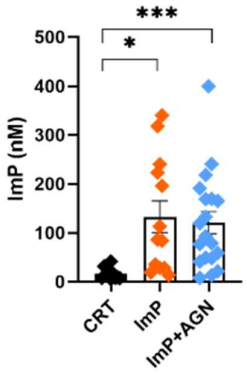


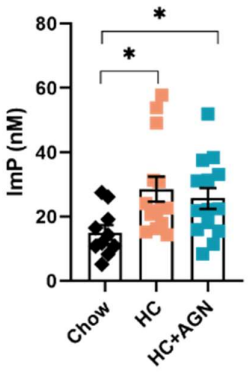
Fig. 13 for the Reviewers. ImP was administered (ImP) or not (CTR) in drinking water to wild type mice fed chow diet for 23 weeks, followed by sacrifice and analysis. **a**, Quantification of atherosclerotic lesion by oil red O en face staining of the aorta showing quantification in whole aorta (left), aortic arch (middle) and representative images (right). n=8-10. Individual data and mean ± SEM of at least two pooled independent experiments. Unpaired Student's t test. ns: not significant.

7. Fig4 and Extended Fig 7. AGN192403 and ImP are provided together in drinking water. Does ImP reach the same levels when provided alone and when provided with this drug?

Following the Reviewer's request, we quantified the concentration of ImP in plasma samples from *ApoE*$^{-/-}$ mice following different treatments: 1) ImP supplementation (400µg/mouse/day) or co-administration of ImP and AGN192403 (AGN, 30µg/mouse/day) in the drinking water to chow-fed mice for 8 weeks (**New Extended Data Fig. 7d)**; 2) AGN192403 treatment (from week 5 to 8) to mice fed HC diet for 8 weeks (**New Extended Data Fig. 8b**). In both models, we observed a statistically significant increase in plasma ImP concentration upon ImP administration or HC diet compared to chow-fed mice ($p<0.05$), and this increase remained unchanged upon AGN treatment.



New Extended Data Fig. 7d. Plasma ImP from *ApoE*$^{-/-}$ mice administered or not (CTR) in the presence of AGN192403 (AGN) or not (ImP) in drinking water to chow-fed mice for 8 weeks. Individual data and mean ± SEM from two pooled independent experiments. Brown-Forsythe and Welch ANOVA with Dunnett's Post Hoc test *$p < 0.05$.



New Extended Data Fig. 8b. Plasma imidazole propionate (ImP) from *ApoE*$^{-/-}$ mice fed chow or high cholesterol (HC) diets for 8 weeks. At 4 weeks post diet initiation, AGN192403 was administered (AGN) or not in the drinking water to mice fed HC diet until week 8. Individual data and mean ± SEM from two pooled independent experiments. Brown-Forsythe and Welch ANOVA with Dunnett's Post Hoc test ***p < 0.005, *p < 0.05

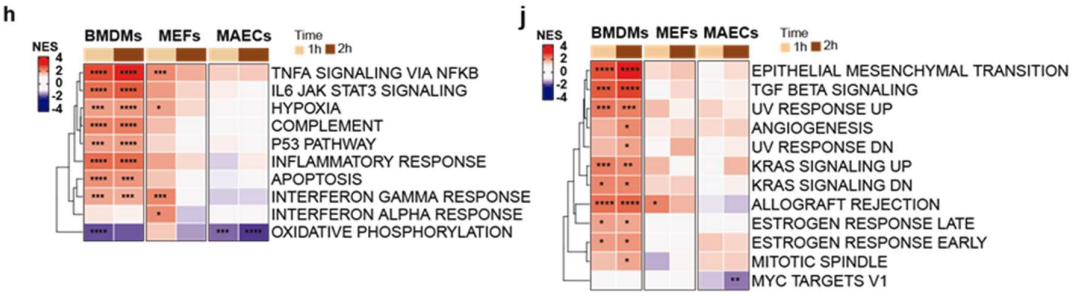
8. Previous work suggested that ImP induces activation of p38γ and subsequent activation of 2 distinct downstream signaling pathways, p62-mTORC1-S6K130 and AKT-AMPK leading to insulin resistance. Please discuss how findings presented in this study are connected with this previous work.

Our new results show that ImP induces inflammatory pathways particularly in BMDMs and MEFs (**New Fig. 3h and Extended Data Fig. 5j**). We hypothesized that ImP-triggered mTOR activation could be crucial for induction of a proinflammatory profile that contributes to atherosclerosis progression, since it is well established that mTOR activation contributes to atherosclerosis progression. Indeed, rapamycin (mTOR inhibitor) prevents atherosclerosis development (https://doi.org/10.1034/j.1600-6143.2003.00094.x, https://doi.org/10.1016/j.atherosclerosis.2003.09.003, https://doi.org/10.1097/01.fjc.0000177985.14305.15, https://doi.org/10.1590/S0100-879X2009005000036, https://doi.org/10.1111/j.1476-5381.2008.00080.x, https://doi.org/10.1186/s12929-016-0274-z, https://doi.org/10.1016/j.intimp.2017.02.026, https://doi.org/10.1074/jbc.M117.779447).

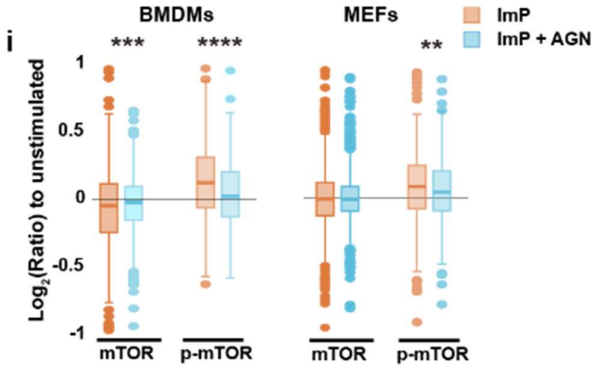
To address this interesting question from the Reviewer, we have performed a proteomics and phosphoproteomics analysis in MEFs and BMDMs. This study revealed increased phosphorylation in peptides corresponding to proteins related to activation of the mTOR pathway (**New Fig. 3h and New Extended Data Fig. 5j**). Since ImP administration induces pS6 and TNFα production inhibited by rapamycin (**Extended Data Fig. 5l,m**) these results suggest that ImP induces an inflammatory profile through

activation of the mTOR pathway. This is consistent, as commented by the Reviewer, with previous studies describing increased activation of this pathway in the liver of patients with type 2 diabetes mellitus (https://doi.org/10.1016/j.cell.2018.09.055). Of note, our results also show that AGN192403 blockade of I1R prevents the activation of this pathway, as demonstrated by our phosphoproteomics studies (**New Fig. 3i**) and results in pS6 and TNFα production (**Extended Data Fig. 6a,b**).

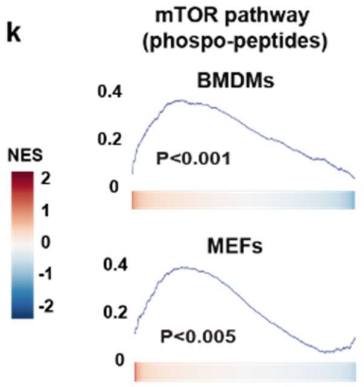
Thus, to get further insights and address the specific contribution of mTOR in myeloid cells to the development of ImP-induced atherosclerosis, we generated *Lyz2*Cre x *Raptor*$^{f/f}$ (*Lyz2ΔRaptor*) mice with impaired mTOR activation in the myeloid compartment. We transplanted BM from *Lyz2ΔRaptor* or control *Raptor*$^{f/f}$ mice to *Ldlr*$^{-/-}$ recipients that were treated (ImP) or not (CTR) with ImP (400µg/mouse/day) in the drinking water. The results showed that Raptor deficiency in the myeloid compartment completely prevented the development of atherosclerosis by ImP (**New Fig. 3j and Extended Data 5o**), demonstrating the key role of mTOR in myeloid cells in this process.



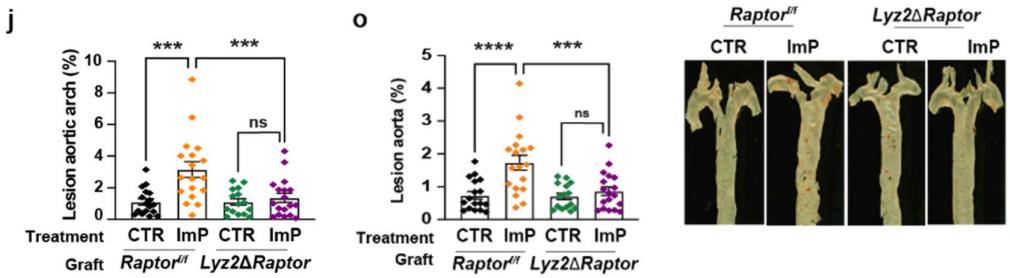
New Fig. 3h.(left) and New Extended Data Fig 5j (right). Heat map of gene-set enrichment analysis from RNAseq analysis comparing ImP stimulation after 1h and 2h with unstimulated within each cell type. Nominal p-values calculated using a 1000-permutation test. * p<0.05; *** p<0.005; **** p<0.001.



New Fig. 3i, Comparison of the global abundance of peptides (mTOR) and phosphopeptides (p-mTOR) from the mTOR pathway in BMDMs (left) or MEFs (right) upon treatment with ImP for 3h in the presence or not of AGN192403. Peptides and phosphopeptides are quantified as Log2 ratio and referred to unstimulated condition. Wilcoxon Signed Rank comparing ImP vs ImP + AGN. *** p<0.005; **** p<0.001.



New Extended Data Fig. 5k. Gene Set Enrichment Analysis comparing phosphopeptides and peptides related to the mTOR pathway after ImP stimulation in BMDMs (top) and MEFs (down). Nominal p-values are calculated using a 1000-permutation test.



New Fig. 3j and Extended Data Fig. 5o. ImP was administered (ImP) or not (CTR) to *Ldlr*$^{-/-}$ mice grafted with BM from control *Raptor*$^{f/f}$ or *Lyz2ΔRaptor* and fed chow diet for 12 weeks. Quantification of atherosclerotic lesion by oil red O en face staining of the aorta showing quantification in aortic arch **(j)** and in whole aorta and representative images **(o).** n=15-18. Individual data and mean ± SEM of at least two pooled independent experiments. Unpaired Student's t test comparing CTR and ImP; One-way ANOVA with Tukey post-hoc correction. *** $p < 0.005$; **** $p < 0.001$.

Other comments

9. "Plasma ImP concentration positively correlated with gut microbiota genera of Escherichia/Shigella and Eubacterium" What is the significance of this finding? Are bacteria in these genera known to make ImP?

This is an excellent question but the resolution of the data does not allow speculation on which bacteria are the main producer of ImP since this would entail metatranscriptomics, enzyme activity measurements as well as isolation of the strains

associated with increased ImP concentrations, followed by cloning of UrdA and enzyme characterization. Our data suggest that the dietary intervention alters microbial ecology with increased levels of Eubacterium and Escherichia/Shigella. However, the resolution of the data is too low to suggest these as producers since the capacity to produce ImP appears to be strain-specific. Accordingly, this would encompass a full new project isolating a diverse assembly of organisms, testing the ability to produce ImP followed by cloning and enzyme characterization. This will take several years, if performed appropriately, and we thus consider it outside the scope of the current manuscript.

10. "16S-based metagenomic analyses identified ImP to be positively associated with the relative abundance of Veillonella, Acidanaminococcus, Actinomyces and Megasphaera, and inversely with Erysipelotrichaceae and Coriobacteriaceae families (Fig. 2d), which were previously described to be altered in CVD subjects"

Human study is a completely different set of bacteria. Do bacteria in these families make ImP? Also, I suggest using the term 16S rRNA gene sequencing instead of 16S-based metagenomic

We agree with the Reviewer and have changed to 16S rRNA gene sequencing throughout the paper. We were not surprised that bacteria differ between mice and humans, which is consistent with our previous findings (https://doi.org/10.1194/jlr.M072819). We have demonstrated that several strains and species of bacteria representing numerous genera have the capacity to produce (https://doi.org/10.1016/j.cell.2018.09.055) or are associated with ImP production (https://doi.org/10.1016/j.cell.2018.09.055 and https://doi.org/10.1038/s41467-020-19589-w) in humans. However, the ability to produce ImP appears to be strain dependent e.g. strains within a single species such as *Lactobacillus gasseri* may or may not produce ImP (preliminary data).

11. Figure 1 h (right) is confusing. Why same genera from different treatment groups adds 100%? Not clear what the point of this figure is

We agree with the Reviewer that Fig. 1h is confusing and we decided to remove the stacked bar plots. The stacked bar plots showed the relative abundance of 16S rRNA sequences at the genus level (>0.1% of total abundance) that were differently enriched in cecal samples from *ApoE*$^{-/-}$ mice fed chow, HC and HC/HC diets for 8 weeks. We agree that how we depicted this data was confusing and did not add any information. Thus, for a matter of clarity we have just removed it from Fig. 1.

12. A very recent study identified ImP associated with atherosclerosis among women living with or at risk of HIV. Please cite

Thank you for the comment. We have now expanded the discussion to also include recent studies on the relationship between ImP and other diseases (lines 244ff).

Point by point response

We appreciate that the Reviewers found our work of significant interest, and we would like to thank them for their helpful and constructive comments. We have addressed all the issues raised by the Reviewers (and also discussed with the Editor) in the revised version of the manuscript, as explained below. Changes to the main text can be tracked in the marked copy. In our point-by-point response, we refer to new Figures corresponding to newly added experiments in the manuscript (highlighted as new). We also provide additional Figures for the Reviewers' attention that, in our opinion, are not strictly necessary to be present in the manuscript. However, we are happy to include any of these Figures/data upon the suggestion of the Reviewers or the Editor.

Reviewer #1

Thank you for your thoughtful responses to my many comments. The manuscript is considerably improved.

We thank the reviewer for the constructive criticisms that have helped to improve the quality of the manuscript.

Reviewer #2

The new submission of the revised version of the manuscript has some commendable improvements.

We thank the reviewer for the constructive criticisms that have helped to improve the quality of the manuscript.

However, the results stemming from the mouse models of atherosclerosis remain unconvincing. I asked for plaque quantifications (for all the experiments), in the aortic sinus, which is an important and classically investigated location in atherosclerosis-related studies. The authors provided unconventional quantifications as they provided a percentage (of what?) of Masson's trichrome staining whereas it was supposed to be a surface of plaques stained by Oil Red O (to visualize lipids).

As requested by the reviewer in the first round of review, we measured atherosclerosis in the aortic sinus. To quantify the damage while also providing detailed insights into tissue architecture, we preferred using Masson's trichrome staining in paraffin-embedded tissue, which allows the quantification of aortic sinus lesion together with a better histological study for plaque composition that was also requested by the reviewer (**Fig. 4i and Extended Data Fig. 4j and k and 8d** in the manuscript). Taking advantage of some experiments where the samples were OCT-embedded, we have now tested Masson's trichrome and Oil Red O staining in parallel in serial sections of the same tissues. Both staining techniques show increased atheroma plaque formation upon ImP supplementation of chow-fed *ApoE*$^{-/-}$ mice (**Fig 1 for the reviewer, new Fig. 3b and Extended Data Fig. 3c**). However, due to the superior quality of sections and the detailed tissue architecture provided by paraffin embedding and Masson's trichrome staining, we preferred this technique to detect and quantify the differences in lesion size. This approach also allowed us to analyse plaque complexity, including necrotic core (**Fig. 4i**) and inflammatory cell accumulation in the aortic sinus plaques (**Extended Data Fig. 4j and k**), as initially requested by the reviewer. The automated analysis of lesion areas in Masson's trichrome staining and the Oil red O staining is not entirely equivalent, since the damaged area detected by the Masson's trichrome exceeds the lipid accumulation detected by Oil red O. However, the significant differences in lesion induced by ImP in atherogenic mice remain, regardless of the method used.

Regarding the quantification, we initially used the % of lesion area relative to whole aortic root, an index that has been previously used in the literature (https://doi.org/10.1038/s41467-023-43896-7; https://doi.org/10.1038/s41419-021-03712-w ; https://doi.org/10.1038/s41598-020-74579-8; https://doi.org/10.1038/s12276-023-00937-x). However, we agree with the reviewer that for lesions in the aortic root, quantifying the extent of the lesion in (μm^2) is more frequent and accurate. Following the reviewer comment, we have recalculated the measurements to indicate the extension of the lesion area as μm^2 and we have added these data in the manuscript (**New Fig. 3b, 4i and Extended Data Fig. 3c**).

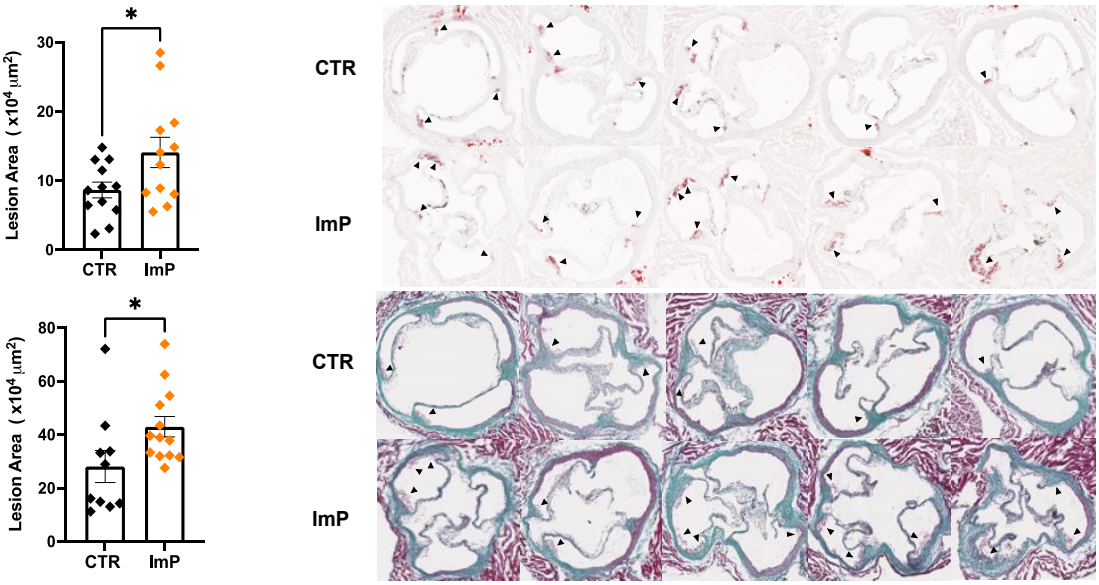


Fig. 1 for the reviewer. *ApoE*$^{-/-}$ mice were administered ImP (ImP) or not (CTR) for 8 weeks. Upper panel. Oil red O staining of the aortic root performed under the indicated treatments. Left: Quantification of atherosclerotic lesions as area of lesion. Right: Representative images. n=12. Lower panel. Masson's trichrome staining of the aortic root performed under the indicated treatments. Left: Quantification of atherosclerotic lesions as area of lesion. Right: Representative images. n=10-13. Individual data and mean ± SEM from at least two pooled independent experiments. Unpaired Student's t test. * $p < 0.05$. This is an extended version for the reviewer of **new Fig. 3b** and **Extended Data Fig. 3c**.

From the figure provided as the new extended Fig 3a, there is a very small plaque in the aortic sinus, and also in the thoracic aorta (for example extended data Fig. 4k, extended data Fig. 5o), which questions the robustness of the quantifications of such small plaques. In this regard and as the authors selected a representative picture for each result and considering the observed small plaques in the aortic sinus, they should provide the pictures of all aortas in the supplement.

As pointed out by the reviewer, the induction of atherosclerosis by administering ImP to hypercholesterolemic mice fed a chow diet is mild, particularly in comparison with the atherosclerosis induced by a high cholesterol diet in these mice. However, it is important to contextualize these results since, as observed in the control mice, both chow-fed *ApoE*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice have very limited lesion at the age when we performed our experiments (8 weeks for *ApoE*$^{-/-}$ mice, and 12 weeks for *Ldlr*$^{-/-}$ mice). Nevertheless, in these hypercholesterolemic mice, ImP alone is sufficient to induce some lesions in the aortic root, and aorta (**Fig. 3a, 3b and Extended Data Fig. 3c,d**) that is prevented upon AGN192403 treatment (**Fig. 4b**). In our opinion, this mild yet measurable induction of atherosclerosis by a single metabolite (ImP) in the drinking water of chow-fed hypercholesterolemic mice, without affecting lipid levels (e.g. cholesterol or triglycerides), is quite remarkable.

Regarding the small lesions generated by ImP in the chow-fed *Ldlr*$^{-/-}$ bone marrow-grafted mice (**New Fig. 3f, 3i, 4a, Extended Data Fig. 4l and 5o**), we would like to

highlight that it is described in the literature, and also shown in our experiments, that irradiation of *Ldlr*$^{-/-}$ mice further reduces atherosclerosis development, which makes this model particularly challenging when using chow-fed mice (https://doi.org/10.1161/hq1001.096724;https://doi.org/10.1161/CIRCRESAHA.119.316539). However, although the induction of atherosclerosis by ImP in *Ldlr*$^{-/-}$ mice fed a chow diet is mild, we chose this model because our unbiased approach demonstrated an important influence of ImP on both the local and systemic immune response in the development of ImP-induced atherosclerosis. Thus, bone marrow grafting of *Ldlr*$^{-/-}$ mice allows for the analysis of the role of immune cell components and genetic modifications, making it a valuable model for exploring underlying mechanisms. Again, under these conditions, we can still detect induction of atheroma plaque by solely adding ImP in the drinking water, allowing us to conclude that ImP-induced atherosclerosis in this model is dependent on the presence of B-T cells (*Rag1*$^{-/-}$ BM), the expression of Raptor in myeloid cells (*Lyz2ΔRaptor* BM) and the expression of Nisch in myeloid cells (*Lyz2ΔNisch* BM) (**New Fig. 3f, 3i, 4a, Extended Data Fig. 4l and 5o**).

Although we initially performed a blinded quantification of lesions using Image J software, we have decided to repeat all measurements with a **standardized automated lesion analysis** based on threshold color analysis to reduce variability and increase reproducibility (https://doi.org/10.3791/59828). Of note, we now include this automated analysis in the manuscript (for **all Figures containing Oil red O staining**) as explained in methods (lines 810ff). The automated analysis corroborates the results included in the previous version of the manuscript by blinded manual analysis.

As requested by the reviewer, **we provide all raw data and quantifications** using this automated method to determine the lesion size for **all Figures of ImP-induced atherosclerosis** as well as some examples showing some representative aortas for the reviewer (**Fig. 2-7 for the reviewer and annexed files with all raw data**). We hope these additional data will convince the reviewer that the differences are statistically significant and consistently demonstrate that ImP supplementation in the drinking water can induce mild atherosclerosis in chow-fed *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, as well as in a post-transplant setting, and that this induction is dependent on the adaptive response, and on the myeloid cell expression of Raptor or I1R. Experiments of bone marrow graft of WT vs *Rag1*$^{-/-}$ have been repeated again to include the proper WT control (**New Fig. 3f and Extended Data Fig. 4l**, **Fig. 6 for the reviewer**) and pictures from aortas were taking again in **Fig. 3i and Extended Data Fig. 5o, Fig. 7 for the reviewer**, to remove the yellow filter used in the previous version.

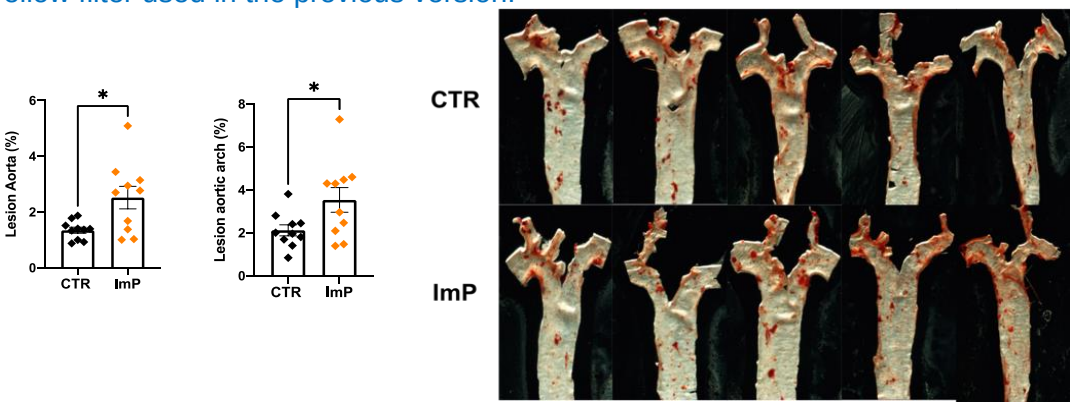


Fig. 2 for the reviewer. ImP was administered (ImP) or not (CTR) in drinking water to *Ldlr*$^{-/-}$ mice fed chow diet for 12 weeks, followed by sacrifice and analysis. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and aorta (up) and representative images (down). n=10. Unpaired Student's t test. * $p < 0.05$; ** $p < 0.01$. This is an extended version of **Fig. 3a and Extended data Fig. 3a** for the reviewer.

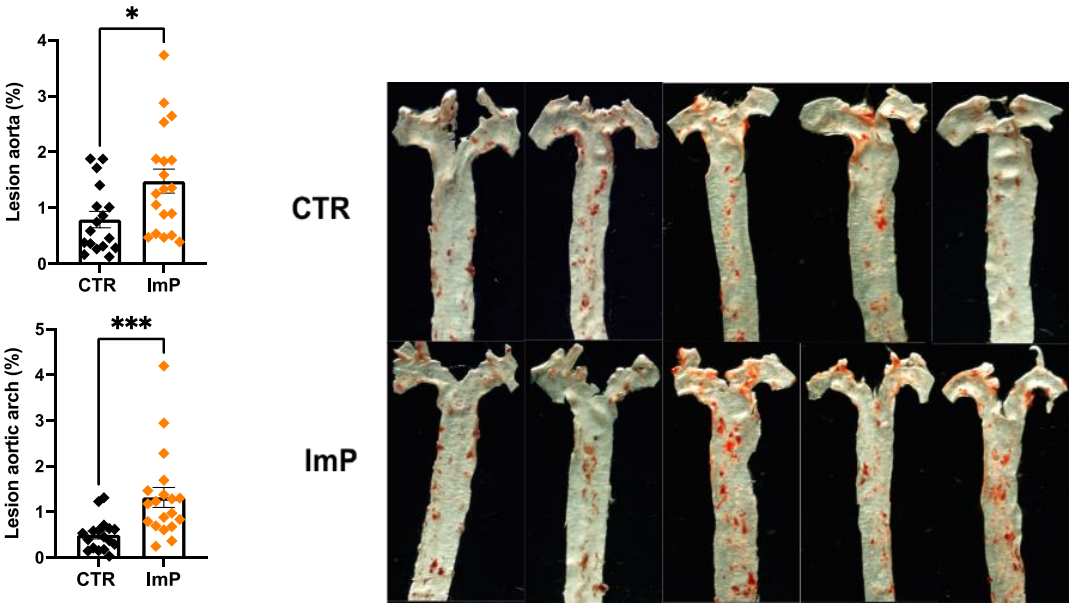


Fig. 3 for the reviewer. ImP was administered (ImP) or not (CTR) in drinking water to *ApoE*$^{-/-}$ mice fed chow diet for 8 weeks, followed by sacrifice and analysis. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and aorta (up) and representative images (down). n=17-19. Unpaired Student's t test. ** $p < 0.01$. This is an extended version of Extended Data Fig**. 3d** for the reviewer.

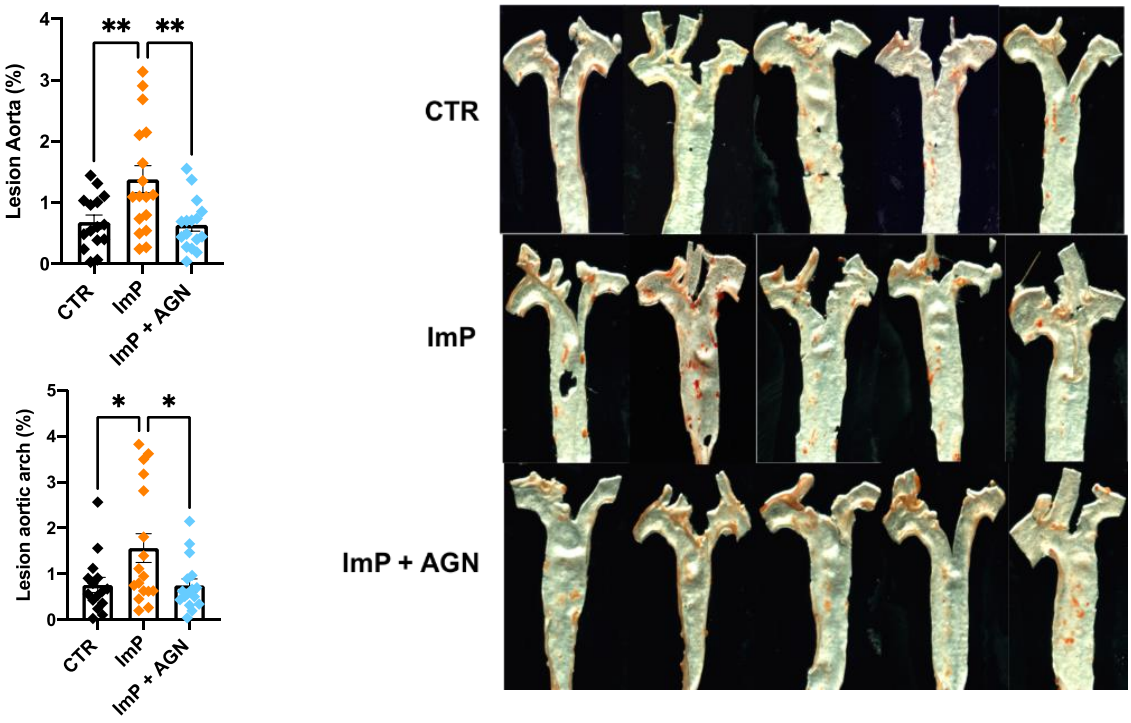


Fig. 4 for the reviewer. ImP was administered (or not, CTR) in the presence of AGN192403 (AGN) or not (ImP) in drinking water to male *ApoE*$^{-/-}$ mice fed chow diet for 8 weeks, followed by sacrifice and analysis. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and aorta (up) and representative images (down) n=15-17. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$. This is an extended version of **Fig. 4b and Extended Data Fig. 7a** for the reviewer.

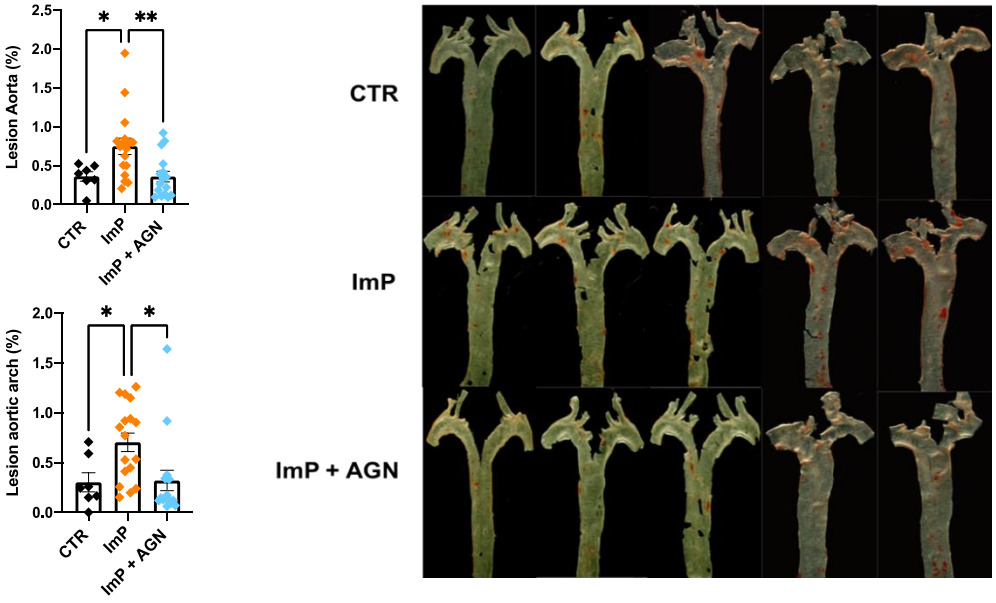


Fig. 5 for the reviewer. ImP was administered (or not, CTR) in the presence of AGN192403 (AGN) or not (ImP) in drinking water to female *ApoE*$^{-/-}$ mice fed chow diet for 8 weeks, followed by sacrifice and analysis. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and aorta (up) and representative images (down) n=7-17. Individual data and mean ± SEM from at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$. This is an extended version of **Extended Data Fig. 7b** for the reviewer.

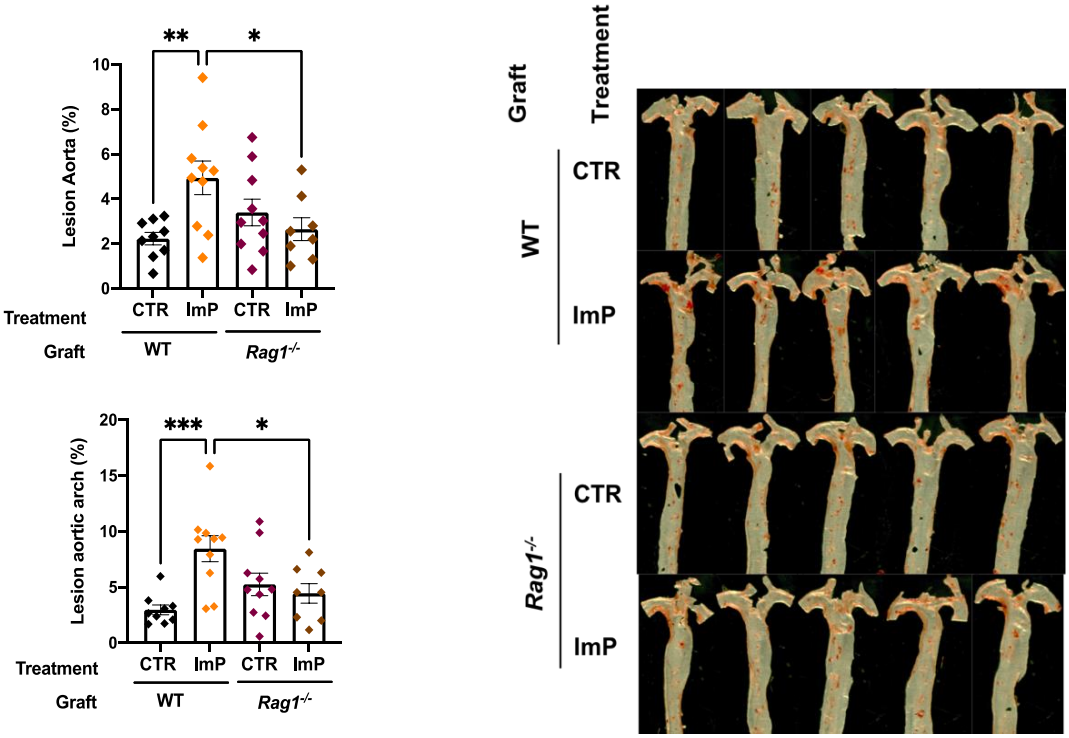


Fig. 6 for the reviewer. ImP was administered (ImP) or not (CTR) to *Ldlr*$^{-/-}$ mice grafted with BM from WT or *Rag1*$^{-/-}$ and fed chow diet for 12 weeks. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and whole aorta (up) and representative images (down) n=8-10. Individual data and mean ± SEM of at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$. This is an extended version of **new Fig. 3f and Extended Data Fig. 4l** for the reviewer.

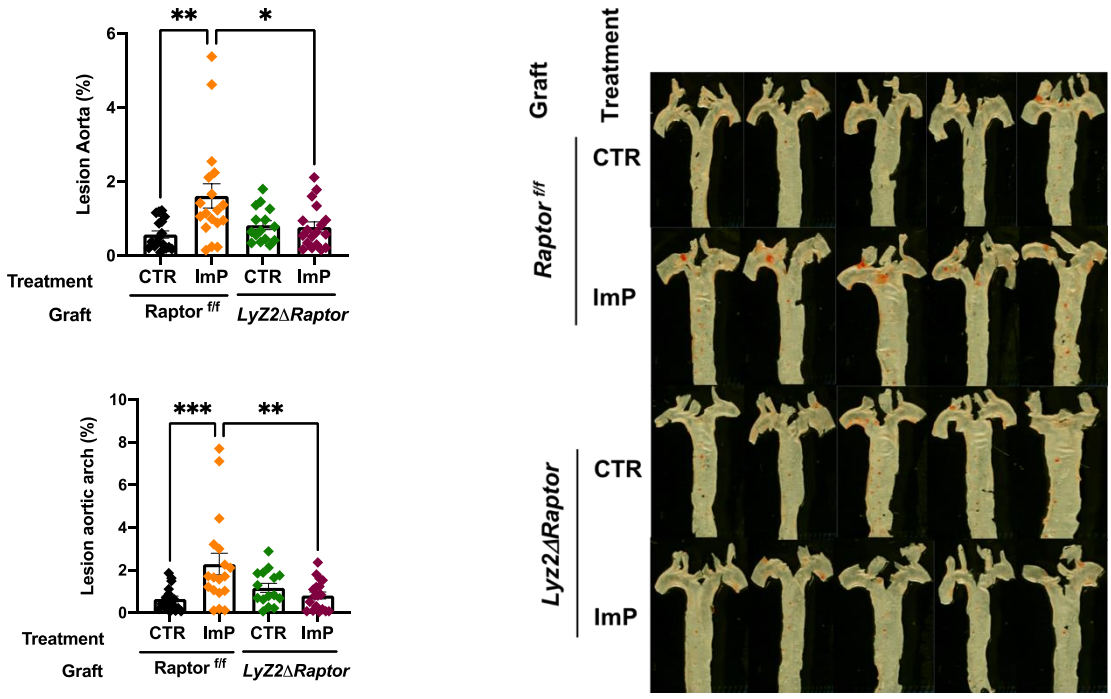


Fig. 7 for the reviewer. ImP was administered (ImP) or not (CTR) to *Ldlr*$^{-/-}$ mice grafted with BM from control *Raptor f/f* or *Lyz2ΔRaptor* and fed chow diet for 12 weeks. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and whole aorta (up) and representative images (down) n=15-18. Individual data and mean ± SEM of at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; **$p < 0.01$; *** $p < 0.005$. This is an extended version of **Fig. 3i and Extended Data Fig. 5o** for the reviewer.

The small size of plaques is because the authors used mice fed CD, which had a low level of blood cholesterol levels and thus small plaques. I understood that the authors "wanted" to present ImP as an earlier marker of atherosclerosis but concluding its role in atherosclerosis based on the results showing such small plaques is not convincing. Having said that, the authors performed an experiment with HC-fed apoe-/- mice in which they only tested the effect of I1R antagonist (shown in Extended Data Fig. 8C) whereas they did not show the effects of ImP supplementation in HC-fed ldlr-/- mice. In any case, the authors should present the impact of ImP supplementation at early and late stages of atherosclerosis development and not only at a very early stage.

We now provide all raw data with automated analysis to quantify atherosclerotic lesions, aiming to demonstrate to the reviewer the mild yet significant induction of atherosclerosis by ImP supplementation in drinking water of chow-fed hypercholesterolemic mice. The rationale for choosing ImP supplementation in a chow diet setting was based on the fact that chow-fed mice have no detectable ImP in their plasma (**Fig. 1**). To establish causality, we believe it is essential to study a clear-cut condition, which is animals without ImP (chow diet) vs animals supplemented with ImP in the drinking water (chow + ImP). As shown in **Fig. 1**, HC diet induces ImP. Thus, if we add ImP to the drinking water in the context of a HC diet, we are comparing mice that naturally produce ImP with those further supplemented with ImP, hindering the study of ImP's effect alone.

In addition, we want to stress that our study is focused on the association of ImP with early atherosclerosis, as also demonstrated in healthy volunteers where we described its association with initial stages of the disease in two independent cohorts. This is particularly relevant since atherosclerosis is a silent precursor to cardiovascular diseases, and its early detection is a critical first step in preventing future complications. Then, of course we agree with the reviewer that studies on the effect of ImP on late stages of the disease can be relevant and very informative, as well as studying the association of this metabolite with subsequent cardiovascular events or the potential effects of pharmacological intervention in late stages of the disease (and plaque regression).

To address the reviewer's concern, we have performed some experiments to dissect whether a further increase in plasmatic ImP in the HC diet context could lead to a further development of the atheroma plaque. We show these new experiments for the reviewer, and we are happy to include them as supplemental data in the manuscript, if required. More in detail, we fed *ApoE*$^{-/-}$ mice chow or HC diet for 4 weeks. In the case of HC-fed mice, they were supplemented or not ImP in the drinking water. We observed that the ImP supplementation increased the average size of atheroma plaque in the context of HC feeding (**Fig. 8 for the reviewer**). This boosting in HC-driven atherosclerosis was even more evident in *Ldlr*$^{-/-}$ mice fed HC diet for 12 weeks and administered ImP in drinking water (**Fig. 9 for the reviewer**). The effect of ImP in the induction of atherosclerosis upon HC diet is also highlighted by the effect of AGN192403 in the reduction of the progression of HC diet-induced atherosclerosis (**Fig. 4h-m and Extended Data Fig. 8**).

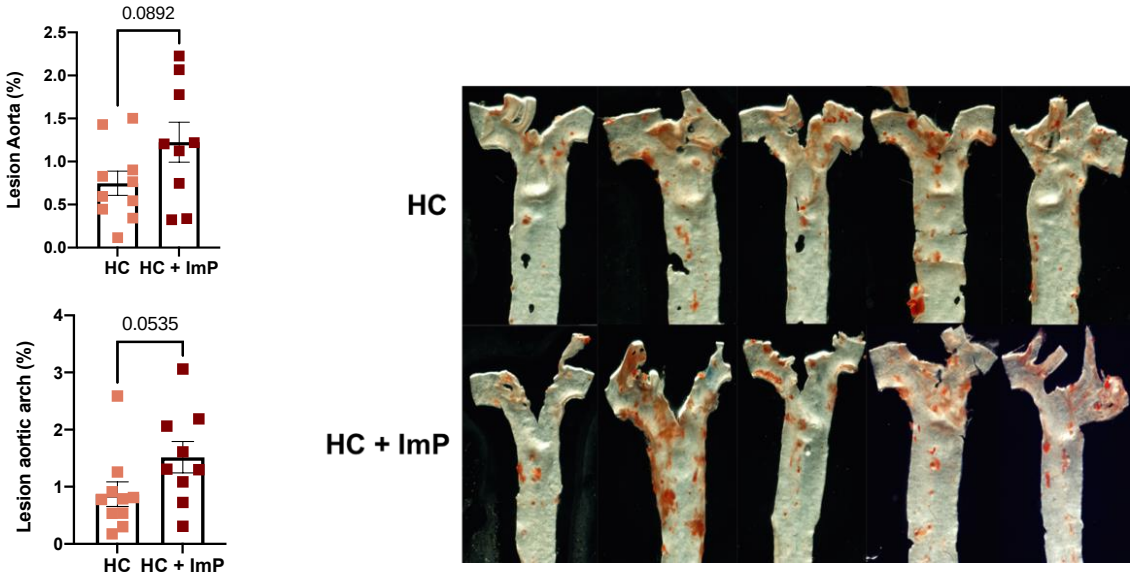


Fig. 8 for the reviewer. ImP was administered (ImP) or not in drinking water to *ApoE*$^{-/-}$ mice fed chow or high cholesterol diet for 4 weeks, followed by sacrifice and analysis. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and aorta (up) and representative images (down). n=13-15. P value for Mann-Whitney analysis comparing both groups is indicated.

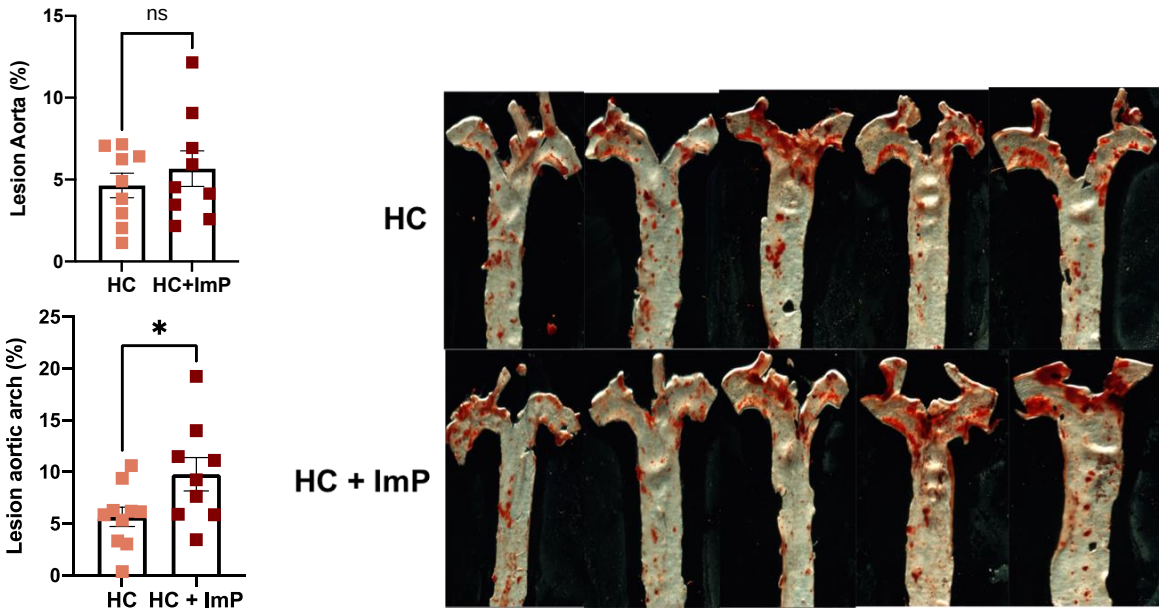


Fig. 9 for the reviewer. ImP was administered (ImP) or not in drinking water to *Ldlr*$^{-/-}$ mice fed chow or high cholesterol diet for 12 weeks, followed by sacrifice and analysis. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and aorta (up) and representative images (down). n=9-10. P value for Mann-Whitney analysis comparing both groups is indicated. *p<0.05; ns, non-significant.

Of note, these data highlighting ImP-induced atherosclerosis in the context of high fat diet administration have been validated in a recently published article (doi: 10.1161/ATVBAHA.124.322346).

The second concern is regarding the in vitro experiments presented in Fig 3H and extended Fig 5J where it is unclear how the results from mouse lines (MAEC and MEF) and in vitro differentiated macrophages (BMDM) could explain the mechanisms involved in vivo? The authors stated “Moreover, RNAseq analysis showed rapid induction of proinflammatory genes in BMDMs and MEFs, but not MAECs, following ImP treatment (New Fig. 3h and New Extended Data Fig 5j), suggesting that MFs and FBs are the main targets of ImP”. Again, for instance, in Fig 3H, the fact that certain mouse lines showed a rapid reactivity 1 or 2 hours after ImP stimulation did not mean that ImP had a preferential impact on these cells in vivo.

We completely agree with the reviewer that any in vitro results should be interpreted with caution. Findings from bone marrow derived macrophages (BMDMs), mouse arterial endothelial cells or mouse embryonic fibroblasts (MEFs) do not necessarily reflect the response *in vivo*. As such we have revised our statements regarding these results using the conditional (suggesting that MFs and FBs could be main targets of ImP, line 211). At any rate, we would like to stress that the objective for the in vitro experiments differed from those performed in vivo. Specifically, with the in vitro experiments, we aimed to improve our understanding of the molecular pathways altered upon ImP stimulation, as well as to identify the receptors potentially involved in its sensing. For instance, **Fig. 3i** shows an increased global abundance of phosphopeptides belonging to the mTOR pathway in BMDMs and MEFs, corroborating previous findings in primary hepatocytes (https://doi.org/10.1016/j.cell.2018.09.055) and allowing us to use pS6 as a readout for the ImP effect in peritoneal macrophages (**Fig. 4e** and **Extended Data Fig. 5n**). Of note, this result is further validated in our in vivo experiment using *Lyz2ΔRaptor* BM grafted

mice indicating the involvement of the mTOR pathway in myeloid cells as essential for ImP-induced atherosclerosis (**Fig. 3i, and Extended Data Fig. 5o**).

To further analyse the function of ImP in myeloid cells we targeted the I1R (*Nisch*) in myeloid cells by mating *Lyz2-Cre* mice with *Nisch*$^{f/f}$ to generate *Lyz2ΔNisch* mice. Analysis of *Nisch* expression showed a selective deletion in spleen myeloid cells compared with CD3^{+} T cells (**New Extended data Fig. 6e**). Notably, the induction of pS6 and TNF production by BMDMs was fully dependent on *Nisch* (**New Extended Data Fig. 6f,g**), thus independently confirming that I1R acts as the host receptor for ImP for these readouts. Once validated the genetic and functional deletion of I1R in myeloid cells in this system, we obtained CD45.2 bone marrow of the *Lyz2ΔNisch* mice or control *Nisch*$^{f/f}$ mice and grafted CD45.1 *Ldlr*$^{-/-}$ recipient mice fed chow diet. Following efficient bone marrow reconstitution 4 weeks later, recipient mice were treated or not with ImP for 12 weeks. En face Oil red O staining of the aorta demonstrated that ImP induction of atherosclerosis is fully prevented when I1R was specifically deleted in myeloid cells (**Fig. 10 for the reviewer and New Fig. 4a and Extended Data 6h**). This result establishes a key role for I1R expression in myeloid cells for the induction of ImP-dependent atherosclerosis. This of course without ruling out ImP effects in additional cell types that could also contribute to boost atherosclerosis.

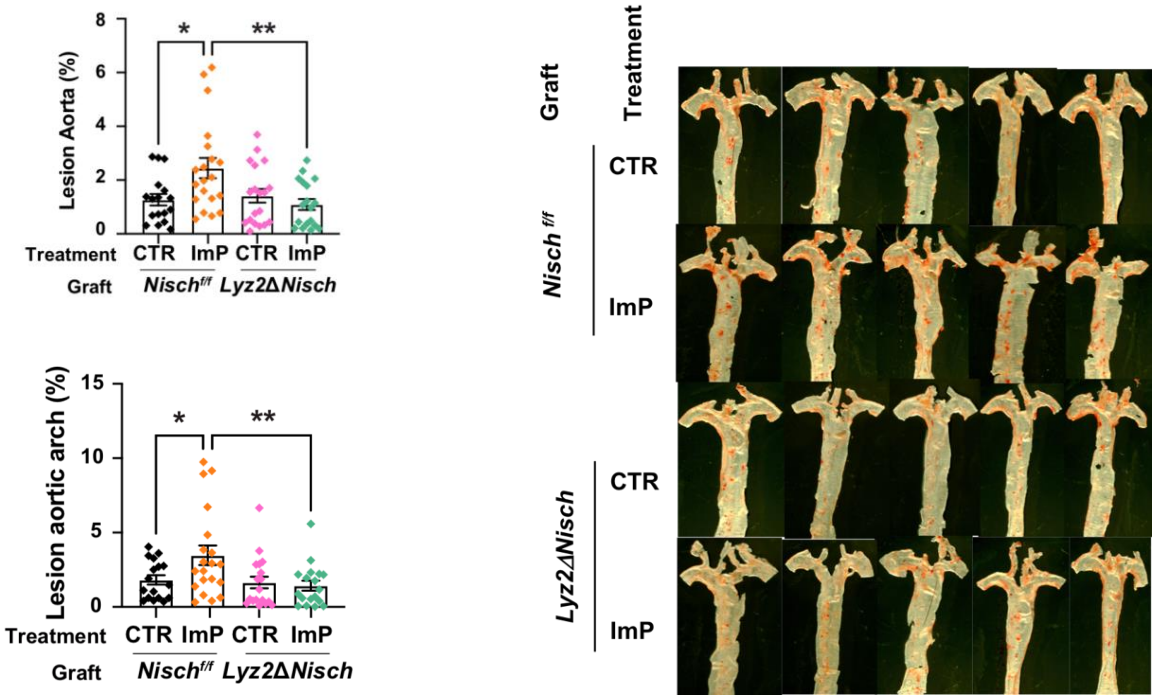


Fig. 10 for the reviewer. ImP was administered (ImP) or not (CTR) to *Ldlr*$^{-/-}$ mice grafted with BM from control *Nisch*$^{f/f}$ or *Lyz2ΔNisch* and fed chow diet for 12 weeks. Oil red O en face staining of the aorta. Graphs show automated quantification of atherosclerotic lesion in aortic arch and whole aorta (up) and representative images (down) n=17-20. Individual data and mean ± SEM of at least two pooled independent experiments. One-way ANOVA with Tukey post-hoc correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$

Moreover, my comment regarding the absence of the link between bacteria and ImP remains. In my opinion, this is an important aspect insofar as ImP production is dependent on microbiota and it is well known that gut microbiota has a huge impact on atherosclerosis development.

The link between ImP production and the microbiota is established from **Fig. 1f**, where we show that the antibiotic treatment blunts ImP levels in plasma, establishing a clear-cut association, as also acknowledged by the reviewer. In addition, the specific generation of ImP by the microbiome is well established in previous literature, along with ImP production by strains from many species (https://doi.org/10.1038/s41467-020-19589-w ; https://doi.org/10.1016/j.cell.2018.09.055). While we understand that establishing the relationship of ImP with specific species or strains of microbiota is of high interest, we believe that this is a distinct objective that will require future, dedicated studies. Our study is rather focused on the association of ImP with atherosclerosis, its causality in the disease, the host sensing axis, and a potential therapeutic intervention by blocking the ImP/I1R axis.

Reviewer #3

The authors addressed adequately all my concerns. Addition of new human cohort plus additional mechanistic studies have strengthen the manuscript.

We thank the reviewer for the constructive criticisms that have helped to improve the quality of the manuscript.

Referee #5 (Remarks to the Author):

This is an interesting and comprehensive report of series of studies from animal model, in-vitro studies, to observational population cohort studies. The focus of my review is on statistical methods and two observational cohorts as instructed by the editors.

The statistical analysis methods used for the data from the PESA and IGT appears to be appropriate and the presentations of the results in the tables and test are sound. Most of the interpretations of the findings are accurate.

We would like to thank the reviewer for his/her thorough review of our work and the positive feedback provided. We are pleased that you found appropriate and sound the statistical methods used for the PESA and IGT cohorts as well as the presentation and interpretation of our findings.

The major comment I have is about the ImP results in table 1a and 2a. I believe that these are raw and unadjusted data (compared with the results in the figures in which adjustments for traditional CVD risk factors were made. However, it is important to see whether the finding is statistically significant in univariate analysis before performing adjustment for potential cofounding factors. The significance seems to be there for the PESA cohort (mean level of 28.7 in the AT group vs 26 in the Control group) as presented in table 1a, however, in the IGT cohort, there is no difference (7 vs 7). Also, not sure how the p value of 0.011 was obtained with the 7 vs 7 comparison.

The reviewer is correct that the values presented in these tables reflect the raw, unadjusted data. As suggested, we agree that it is important to determine whether the findings are statistically significant in univariate analysis before adjusting for potential confounding factors.

In this regard, we performed both the Kolmogorov-Smirnov and Shapiro-Wilk normality tests to assess the distribution of ImP in the two groups (control [CTR] and subclinical

atherosclerosis [AT]). Since the ImP did not follow a normal distribution, we conducted the Mann–Whitney U test to compare ImP between the two groups, ensuring an appropriate non-parametric analysis. The p-value reported in the tables corresponds to this test.

These steps confirm that the findings were statistically assessed in a univariate context prior to any adjustment for traditional cardiovascular risk factors, as shown by the other analyses and in the figures. Details of the tests performed for each cohort are provided below (**Tables 1-4 for the reviewer**).

Moreover, we would like to clarify that the data presented for both cohorts in the tables represent the median values, not the mean values, of ImP in each group (CTR and AT), as the distribution of ImP does not follow a normal distribution (**Table 1 and 3 for the reviewer**). While the median value between the two groups does not vary, as it happens for the IGT cohort and noted by the reviewer, the distribution of this variable between the groups is distinct (as shown in **Fig. 2a and 2c**). This is reflected in the statistically significant p-value, which compares distributions of this variable between the two groups rather than the medians directly.

We hope this clarifies our approach and addresses the reviewer's concerns.

Table 1 for the reviewer. Results of normality tests for plasmatic ImP concentration in control (CTR) and subclinical atherosclerosis (AT) subjects from the PESA cohort.

	CTR_{PESA}	**AT_{PESA}**
Shapiro-Wilk test		
W	0.9641	0.6799
P value	0.0061	<0.0001
Passed normality test (alpha=0.05)?	No	No
P value summary	**	****
Kolmogorov-Smirnov test		
KS distance	0.06793	0.1699
P value	>0.1000	<0.0001
Passed normality test (alpha=0.05)?	Yes	No
P value summary	Ns	****
Number of values	105	295

Table 2 for the reviewer. Results of the Mann-Whitney U test for plasmatic ImP concentration in control (CTR) and subclinical atherosclerosis (AT) subjects from the PESA cohort.

P value	0.0125
Exact or approximate P value?	Approximate
P value summary	*
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A, B	18511, 61690
Mann-Whitney U	12946

Table 3 for the reviewer. Results of normality tests for plasmatic ImP concentration in control (CTR) and subclinical atherosclerosis (AT) subjects from the IGT cohort.

	CTR_{IGT}	**AT_{IGT}**
Shapiro-Wilk test		

W	0.4891	0.1529
P value	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No
P value summary	****	****
Kolmogorov-Smirnov test		
KS distance	0.2589	0.3740
P value	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No
P value summary	****	****
Number of values	529	1315

Table 4 for the reviewer. Results of the Mann-Whitney U test for plasmatic ImP concentration in control (CTR, n=529) and subclinical atherosclerosis (AT, n=1315) subjects from the IGT cohort.	
P value	0.0004
Exact or approximate P value?	Approximate
P value summary	***
One- or two-tailed P value?	Yes
Sum of ranks in column A, B	Two-tailed
Mann-Whitney U	451436 , 1249655
AT is assessed by ultrasound imaging of carotid arteries and presence of calcium score.	

In the case of the p-value referring to the comparison of the ImP in the two groups (AT vs CTR) for the IGT cohort in table 2a, we have noticed that the values were taken from a previous analysis. Specifically, in the earlier analysis, the subclinical atherosclerosis group in the IGT cohort was assessed using ultrasound imaging of the carotid arteries. However, in the final manuscript, to maintain consistency with the PESA cohort, we included the presence of coronary artery calcium, assessed by CT scan. This update resulted in a change in the number of individuals within the group: 1006 (previous) vs. 1315 (current), and a corresponding adjustment in the p-value: 0.011 (previous) vs. 0.0004 (current) (**Table 5 for the reviewer**). We apologize for this oversight and have corrected it in the revised manuscript.

Lastly, we are glad to share the raw data with the reviewer, should s/he wish to verify the data and replicate the tests we have reported.

Table 5 for the reviewer. Results of the Mann-Whitney U test for plasmatic ImP concentration in control (CTR, n=838) and subclinical atherosclerosis (AT, n=1006) subjects from the IGT cohort.	
P value	0.0107
Exact or approximate P value?	Approximate
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	744114, 956976
Mann-Whitney U	392573
AT is assessed by ultrasound imaging of carotid arteries.	

One related question to this point is whether the same or similar ImP analytic tools were used in these two cohort studies. It is stated in the manuscript that ImP was confirmed by using the reference standard. The value of 28 nmol in the PESA cohort is 4 times than in the IGT cohort, although I understand that main characteristics of participants in the

two cohorts are different. The difference appears to be too large. It would be helpful to explain this in the manuscript.

We agree with the reviewer's observation about the differences in ImP quantification between the PESA and IGT cohorts. Several factors could help explain these differences. First, it is important to note that currently there is no universal method for the ImP quantification. In our study, two independent laboratories developed and validated methods using liquid chromatography coupled to mass spectrometry and a targeted metabolomics approach with reference standards. While this technique/approach is considered the preferred choice due to its accuracy and sensitivity, the methodologies used differ, as described in the method section, contributing to variations in ImP quantification.

Additional factors that can contribute to variability from both analytical and biological perspectives, include:

- Preanalytical factors. Variations in storage time, thawing/freezing cycles, personnel involved in sample handling.

- Sample derivatization. In the IGT cohort plasma samples undergo n-butyl ester derivatization before injection in the equipment.

- Analytical factors (R&D-focused methodologies). Differences in columns, mobile phases, and standards between the two methods:

    - PESA cohort: polar HILIC column, 20 mM ammonium formate in ultrapure water at pH 3 (A-phase), and 20 mM aqueous ammonium formate at pH 3 in ACN/H20 (9:1, v/v) (B-phase), with unlabeled ImP standard.

    - IGT cohort, apolar C18 BEH column, water with 0.1% formic acid (A-phase) and acetonitrile with 0.1% formic acid (B-phase), with labelled ImP-^{13}C3 standard.

- Participant characteristics. Differences in participant demographics and environmental exposures between cohorts, such as higher proportion of women and slightly older individuals in the IGT cohort.

- Dietary effects/habits. As ImP is microbially produced dietary differences between Spain (PESA) and Sweden (IGT) could influence its plasma concentration.

However, we would like to emphasize that despite all these factors, all statistical analyses showing a significant association between increased ImP and atherosclerosis in one cohort were consistently confirmed in the other cohort.

We have now included a sentence in the manuscript to address this observation (line 288ff).

I have several minor observations for clarification also.

1. Line 125: PCS needs to be defined. Is it Principal Component Analysis?

Thank you for the observation. Yes, PCA stands for Principal Component Analysis. It has been now defined in the manuscript.

2. Line 1054: do PCs mean “Principal Components”? Needs to be defined when it first appears as it does not appear to be a standard acronym.

Thank you for the observation. Yes, PC stands for Principal Components. It has been now defined in the manuscript.

3. Table 1a about Triglycerides: The overall mean value is 87, while the mean for both control and AT groups is 80. Is it a typo? It should not be statistically significant with the comparison between 80 and 80.

Thank you for your comment. We have identified an error in the triglyceride values for the AT group. The correct values should be as follows: the median for triglycerides in the AT group is 91mg/dL, with quartiles 1 and 3 at 68mg/dL and 124mg/dL, respectively. We apologize for this oversight and have corrected the values in the revised manuscript (New Table 1a).

4. Not sure how the P vale of 0.023 was obtained with the HbA1c comparison across 3 tertiles in the IGT cohort (see portion of the table 2b with mean value of 35 for all three groups).

Thank you for your comment. The p-value for the HbA1c comparison across the three tertiles in the IGT cohort was obtained using the Kruskal-Wallis test, as stated in the table footnote. Specifically, since the HbA1c values do not follow a normal distribution in the three groups, we reported the median value, rather than the mean, in the table and applied the appropriate non-parametric test. The p-value presented in the manuscript corresponds to the result of this test that accounts for data distribution. Details of the tests performed are provided below (**Tables 6 and 7 for the reviewer**).

Table 6 for the reviewer. Results of normality tests for HbA1c across ImP tertiles in the IGT Cohort

	HbA1c_Tertile1	HbA1c_Tertile2	HbA1c_Tertile3
Shapiro-Wilk test			
W	0.9664	0.8747	0.9034
P value	<0.0001	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No	No
P value summary	****	****	****
Kolmogorov-Smirnov test			
KS distance	0.07892	0.1318	0.1051
P value	<0.0001	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No	No
P value summary	****	****	****

Table 7 for the reviewer. Results of the Kruskal-Wallis test for HbA1c across the three ImP tertiles in the IGT cohort.

Kruskal-Wallis test	
P value	0.0226
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	7.582
Data summary	

Number of treatments (columns)	3
Number of values (total)	1839

5. About hs-CRP levels: in the PESA cohort (table 1a), the mean in the control group is 0.11 and the AT group is 0.14, which shows a marginal statistically significance, however, in the IGT cohort, the mean vale is 0.10 for the control group and 0.12 for the AT group, but the difference is highly significant. Is it because of larger sample size in the IGT cohort and the PESA cohort is under power or something else?

We would like to clarify that, due to the non-normal distribution of the hs-CRP in the two groups across both cohorts (**Tables 8 and 9 for the reviewer**), the values reported in the manuscript tables represent the median values, rather than mean values. We believe that the observed differences in the significance could be explained by the inter-individual variability (distribution) of the variable within the same cohort. In **Fig. 11 for the reviewer**, we have included plots illustrating the distribution of hs-CRP in the two compared groups (AT vs CTR) for both cohorts. We hope these plots provide further insight into the comparison and address the reviewer's comment.

Table 8 for the reviewer. Results of normality tests for hs-CRP in control (CTR) and subclinical atherosclerosis (AT) subjects from the PESA cohort.

	CTR	**AT**
Test for normal distribution		
Shapiro-Wilk test		
W	0.3438	0.4818
P value	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No
P value summary	****	****
Kolmogorov-Smirnov test		
KS distance	0.3839	0.3159
P value	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No
P value summary	****	****
Number of values	105	293

Table 9 for the reviewer. Results of normality tests for hs-CRP in control (CTR) and subclinical atherosclerosis (AT) subjects from the IGT cohort.

	CTR	**AT**
Test for normal distribution		
Shapiro-Wilk test		
W	0.6889	0.3677
P value	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No
P value summary	****	****
Kolmogorov-Smirnov test		
KS distance	0.2239	0.3112
P value	<0.0001	<0.0001

Passed normality test (alpha=0.05)?	No	No
P value summary	****	****
Number of values	528	1312

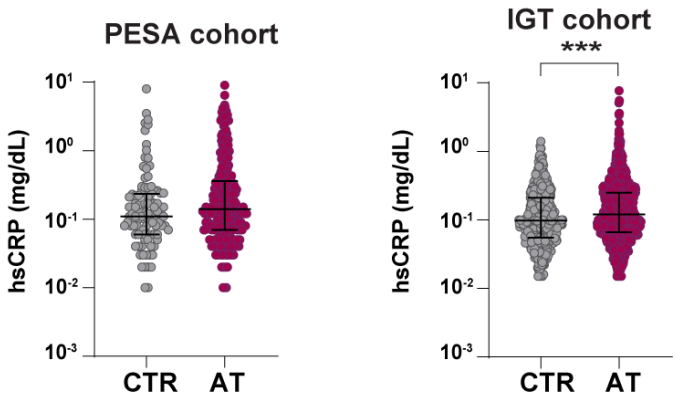


Fig. 11 for the reviewer. Plot showing the distribution of the hs-CRP in controls vs subclinical atherosclerosis volunteers from the PESA (left) and IGT (right) cohorts. Median and interquartile ranges are shown for each group. Mann–Whitney U test. ***p < 0.001.

Point by point response
We appreciate that the Reviewers found our work of significant interest, and we would like to thank them for their helpful and constructive comments. We have addressed all the issues raised by the Reviewers (and also discussed with the Editor) in the revised version of the manuscript, as explained below. Changes to the main text can be tracked in the marked copy. In our point-by-point response, we refer to new Figures corresponding to newly added experiments in the manuscript (highlighted as new).

Referees' comments:

Referee #1 (Remarks to the Author):

None

Referee #2 (Remarks to the Author):

I appreciate the authors' efforts to address my concerns regarding the quantification of small plaques. It is clear that the mouse models utilized by the authors develop very small plaques, and the rationale for using a chow diet to demonstrate that ImP increases atherosclerosis, akin to how a high-cholesterol diet elevates its levels, is well understood.

However, the main issue lies in the threshold. When mice were fed a high-cholesterol diet, they still exhibited pro-atherogenic effects of ImP, albeit with increased ImP levels in both non-supplemented and ImP-supplemented groups. It would be beneficial to include experiments conducted with a high-cholesterol diet in the supplementary figures. Alternatively, using older Apoe-/- mice, which naturally develop plaques as they age and do not require a high-cholesterol diet like Ldlr-/- mice, could provide another viable approach.

Having said that, the authors present convincing images and quantifications throughout their paper to validate the accuracy of their findings.

As requested by the referee #2, we have now included the HC diet experiments that were provided to the referee as supplementary figures (New ED Fig. 3f and ED Fig. 3g) that are commented in the results section (lines 168ff).

Referee #3 (Remarks to the Author):

The authors have adequately addressed my comments. This is a significantly improved version of the manuscript. I commend the authors for the thorough responses.

Reformatting requirements highlighted by the Editor:
In addition to the general formatting requirements in this email, we ask that you please pay particular attention to the following reformatting points:

1. Please submit a revised title within 75 characters (including spaces) that is free of any punctuation marks like colons, exclamation marks, full stops or speech marks. OK
2. Flagging that methods references are not continuously numbered. Corrected
3. Please remove the main figures from the article file and re-supply them individually in an acceptable format such as EPS, AI, PS, PDF, PPT, PSD or XLS (for graphs) with editable vector files. Supplied.
4. Please ensure all main figure legends are 300 words or less. OK
5. Please reduce subheadings to 40 characters (with spaces) or less. OK

6. Flagging that there are potential third party rights issues in the figures - please check sources or if permissions are needed for the mice, human silhouette illustrations in the figures. No third party right issues required.
7. Flagging that there are potential third party rights issues in the figures and the TPR table has not been provided on eJP. TPR table completed.
8. Please remove the Extended data figures from the article file and re-supply them individually in EPS, JPEG or TIF format. Done.
9. Please ensure that the text size in all figures is at least 5 pt Arial. Checked and corrected.
10. Please put the data regarding the HFHC diet into the ED Figures as suggested by reviewer #2. Added as new ED Fig. 3f and 3g (see response to reviewer and comments in results, lines 168ff).
11. Please ensure that all data (metabolomics, scRNA-seq, proteomics, bRNA-seq, phosphoproteomics) are deposited. All the data are deposited. We provide reference for bulk RNA-seq, proteomics and phosphoprotomics. Metabolomics data are deposited and we have data track ID, but the reference will be provided upon validation. scRNAseq data are deposited and also expecting validation to provide the final reference.
12. Please move all tables from Extended DAta to Supplementary Information. Tables moved as supplementary information with format that can be edited as requested.

We have also followed the general formatting requirements (see below) and we are ready to address any other issues that may arise.

Paper editing for Nature publishing

1. Please submit a revised title within 75 characters (including spaces) that is free of any punctuation marks like colons, exclamation marks, full stops or speech marks.

Host sensing of microbially produced imidazole propionate is a driver and therapeutic target in atherosclerosis 111/75

We provide a new title: Imidazole propionate is a driver and therapeutic target in atherosclerosis

2. Flagging that methods references are not continuously numbered.

This has been addressed in the manuscript.

3. Please remove the main figures from the article file and re-supply them individually in an acceptable format such as EPS, AI, PS, PDF, PPT, PSD or XLS (for graphs) with editable vector files.

We provide main figures as pdf.

4. Please ensure all main figure legends are 300 words or less.

Corrected in the manuscript

5. Please reduce subheadings to 40 characters (with spaces) or less.

Corrected in the manuscript

6. Flagging that there are potential third party rights issues in the figures - please check sources or if permissions are needed for the mice, human silhouette illustrations in the figures.

We have checked that there are no third party right issues and the figures are original.

7. Flagging that there are potential third party rights issues in the figures and the TPR table has not been provided on eJP.

Figures are original and we provide the completed table.

8. Please remove the Extended data figures from the article file and re-supply them individually in EPS, JPEG or TIF format.

Now they are provided as JPEG.

9. Please ensure that the text size in all figures is at least 5 pt Arial.

Figures corrected to assure at least this size for letters.

10. Please put the data regarding the HFHC diet into the ED Figures as suggested by reviewer #2.

Added as new ED Fig. 3f and 3g (see response to reviewer and comments in results, line 168ff).

11. Please ensure that all data (metabolomics, scRNA-seq, proteomics, bRNA-seq, phosphoproteomics) are deposited.

All the data are deposited. We provide reference for bulk RNA-seq, proteomics and phosphoproteomics. Metabolomics data are deposited and we have data track ID, but the reference will be provided upon validation. scRNAseq data are deposited and also expecting validation to provide the final reference.

12. Please move all tables from Extended DAta to Supplementary Information

Tables moved as supplementary information.

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STATISTICS: When revising your manuscript, you should ensure that any statistical analysis used is sound and that it conforms to our guidelines. A collection of articles explaining the basics of statistical analysis and advice on how to best present it can be found here.

We have revised the statistics.

REPRODUCIBILITY: To ensure that the quality and transparency of methods and statistical reporting (as discussed here) are sound before the paper is published, we have reviewed your Reporting summary and Editorial policy checklist editorially. I have attached two documents: one listing specific issues related to your manuscript and one containing an annotated version of the Reporting summary. Please ensure that, as well as the more general points below, the points highlighted in the attached documents are addressed in full, both on these forms and within the manuscript. Both forms should be uploaded as a "Related Manuscript" file type. The Reporting summary will be published with your paper.

We have addressed the points raised and uploaded the reporting summary and the editorial request table as related manuscript files.

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In this case, we feel the current length of the paper is appropriate, so no further shortening is necessary; you should not significantly add to the text when revising.

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Corrected

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MAIN TEXT: If further introductory material is necessary, the main text can begin with up to 500 words of introduction expanding on the background to the work (some overlap with the summary is acceptable), before proceeding to a concise, focused account of the findings, and ending with 1 or 2 short paragraphs of discussion. Sections are separated with subheadings (up to 40 characters including spaces) to aid navigation.

REFERENCES: As a guideline, most papers should include no more than 50 main text references; all additional references can be cited in (and listed after) the Methods section, as

detailed below.

FIGURE LEGENDS: These should be listed sequentially after the main text references and not in the figure files. Each legend should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used. Legends should not exceed 300 words each. Each figure legend should contain, for each panel where relevant, the following information:
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* a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc);
* a statement of how many times the experiment shown was replicated;
* definitions of statistical methods and measures:
* very common tests (e.g. t-test, simple Chi-square tests, Wilcoxon and Mann-Whitney tests) can be identified by name only, but more complex techniques should be described in the Methods;
* whether tests are one-sided or two-sided;
* whether there are adjustments for multiple comparisons;
* the statistical test results (e.g., P values);
* the definition of 'center values' as median or average;
* the definition of error bars as s.d. or s.e.m.
Descriptions that are too long for the figure legend should be included in the Methods section.

All these concerns have been corrected.

METHODS: The Methods section, which provides the full, step-by-step instructions that would allow other researchers to replicate the results, is included after the main text figure legends. The Methods section will not appear in print but will appear online in the full-text HTML and PDF versions. The Methods section should be written as concisely as possible but should contain all elements necessary to allow interpretation and reproduction of the results. If there are additional references (in the Methods section, Supplementary Information, etc), their numbering should continue from the last entry in the main text reference list, and they should be listed following the Methods section. Specialized methods that require chemical structures, figures, or tables cannot be accommodated in the Methods section of the main text file. If such information is part of the Methods, the entire Methods section must instead be included within a Supplementary Information text file.

MAIN TEXT STATEMENTS: Several statements (which will not appear in print but will appear online in the full-text HTML and PDF) are required after the Methods (and additional references, if present). First, there should be an Acknowledgements section, listing grant/financial support. Next, we require a detailed Author Contribution statement; the specific contributions of each author, particularly in terms of which authors performed which specific experiments, must be listed. This is followed by a Competing Interest statement. Financial and non-financial interests should be noted here, as well as any patents; patent information should include at a minimum patent number, what is covered by the patent, and who submitted the patent application. Finally, an Additional Information statement should include information regarding reprints and permissions and name the author(s) to whom correspondence and requests for materials should be addressed. Formatting details and an example are available here.

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Extended data provided at the end of the main text.

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CELL LINE IDENTIFICATION: To help curb the inadvertent use of cross-contaminated or misidentified cell lines, we ask that you check your reagents against the list of commonly misidentified cell lines maintained by the International Cell Line Authentication Committee, which is also accessible through the NCBI BioSample database. If you have used a cell line that is on this list, you must provide a scientific justification and state the identity issue in the Methods. The editors reserve the right to demand that the data be removed from the paper if the justification is deemed unsatisfactory. In addition, authors must identify the source of cell lines (with catalog number if obtained from a vendor or cell bank) and report whether the cell lines have been authenticated, including the method used, the results, and the date authentication testing was last performed for that cell line. You should be able to provide the test results upon request. Mycoplasma contamination testing status must also be reported. These requirements will be particularly scrutinized for cancer studies, where the issue of cell line misidentification has been well documented. Resources on cell line authentication are available here.

SOURCE DATA (GRAPHS): To increase transparency, we strongly encourage you to provide, in spreadsheet form, the data underlying the graphical representations used in figures. For all experiments presenting data from animal models, this is a requirement and is not optional. This is in addition to our well-established data-deposition policy for specific types of experiments and large datasets. Online readers of the manuscript will be able to access the graphical source data directly from the figure legend. Spreadsheets must be submitted in .xls, .xlsx or .csv formats. One file per figure is permitted. If there is a multi-panelled figure, the source data for each panel should be clearly labeled in the file; alternatively the source data for a figure can be included in multiple, clearly labeled sheets within an Excel file. File sizes of up to 30 MB are permitted, but it is expected that the vast majority of graphical source data files will be considerably smaller than this. When submitting these files with your manuscript, you should select the "Source Data" file type and use the title field in the file description tab to indicate the figure(s) to which the source data pertain. Source data should not be provided as Extended Data.

RAW DATA (GELS): You must provide the original source images for all data obtained by electrophoretic separation (e.g., EMSA, northern/Southern/western blots, etc). The raw images must be assembled into a single .pdf or .tif file (multiple gels on a single page is encouraged). The file should be uploaded as Supplementary Figure 1. The full scanned images must be in uncropped form and contain labeled size/molecular weight markers and loading controls. There should be an accurate indication of how the gels were cropped for the final figure. The figure legends and raw data files should indicate whether controls (such as beta-actin) were run on the same gel as loading controls, or on separate gels as sample processing controls (see here for guidance). While the data can be displayed in a relatively informal style, there must be a correspondence between each source data image and a specific main text or Extended Data figure. The main text or Extended Data figure legends should refer to the uncropped scans explicitly (e.g., “For gel source data, see Supplementary Figure 1.”). For examples,

see here or here.

DATA DEPOSITION: The following specific points may be relevant to your paper, so please ensure that you provide the following information:

* MASS SPEC DATA: For any metabolomics, lipidomics data or other mass spec-based data, we ask that you adhere to current best practices in terms of data reporting. For this, we ask that you please deposit your mass spec data in a public repository, such as MetaboLights (https://www.ebi.ac.uk/metabolights/), Metabolomics Workbench (metabolomicsworkbench.org), or MASSIVE (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsprun), or one of the repositories suggested by Scientific Data (https://www.nature.com/sdata/policies/repositories#life). This is meant to help to improve reproducibility and data sharing in the field. In addition, we ask that you include all spectral data used to identify metabolites used in hypothesis generation. This can be provided as supplementary information. Please see these metabolomics (https://www.nature.com/articles/s41592-021-01197-1) – see Box 1–and lipidomics (https://www.nature.com/articles/s42255-022-00628-3) guidelines for minimal reporting standards of sample prep, run, analysis, and machine set-up.

* Sequences for any RNAi/small RNA constructs must be included.

* Accession numbers for gene expression data or RNA sequencing data must be listed.

* Papers reporting protein structures must conform to our standards listed in the Guide to Authors. The Data Availability statement must state that the X-ray crystallographic coordinates and structure factor files (or comparable NMR or cryoEM data) have been deposited in the appropriate, named, public database, along with all relevant accession number(s). You must use the standard Nature templates for structural data; there are separate links to tables for X-ray crystallographic, NMR and cryoEM structures. These tables must be presented as Extended Data; if the number of entries causes the table to exceed a page, it must be divided into two Extended Data items. The contour level of any electron density maps presented, as well as the type of map (i.e., Fo-Fc or 2Fo-Fc), should be explicitly stated in the figure legend.

* For every new chemical compound, a complete description of the synthesis and the physical characterization (i.e., NMR, MS, etc) must be included in the Supplementary Information (see here).

* Papers containing new or revised formal taxonomic nomenclature for animals, whether living or extinct, are accepted conditional on the provision of LSIDs (Life Science Identifiers) by means of registration of such nomenclature with ZooBank, the online registration system for the International Code of Zoological Nomenclature (ICZN). ZooBank LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/".

* We strongly encourage deposition of 3D morphological data in a suitable repository such as MorphoBank, MorphoSource or similar; the relevant accession numbers should be listed in the Data Availability statement.

* For animal experiments, you must confirm that all experiments were performed in accordance with relevant guidelines and regulations. There should be a statement identifying the institutional and/or licensing committee approving the experiments, including any relevant

details. Sex and other characteristics of animals that may influence the results must be described. Details of housing and husbandry must be included if they are likely to influence experimental results. Further details can be found here.

* Human genotype data (e.g., SNP array data) should be deposited into a public database (dbGAP or EGA) with a controlled access policy.

* A full clinical and pathological characterization of patients/human subjects and samples should be provided in tabular format, including the magnitude of response for each patient (partial, complete, stable disease), the site of the biopsy, whether or not that lesion was progressing and mutational status if appropriate.

CONSORTIA: For papers containing one or more consortia, all members of the consortium who contributed to the paper must be listed in the paper (i.e., print/online PDF). If necessary, individual authors can be listed in both the main author list and as a member of a consortium listed at the end of the paper. When submitting your revised manuscript via the online submission system, the consortium name should be entered as an author, together with the contact details of a nominated consortium representative. See here for our authorship policy and here for further consortia formatting guidelines, which should be adhered to prior to acceptance.