

Dietary advanced glycation end-products promote food allergy by disrupting intestinal barrier and enhancing Th2 immunity

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript deals with the dietary contamination of AGE products that are introduced during food processing and the risk on developing food allergy as AGE may act as and type 2 skewing adjuvant. This topic is very relevant and many of the data shown in this manuscript and the supplementary figures are of interest. The paper shows analyses of an observational study, the effects of an AGE rich diet in three different ovalbumin induced murine models. These contain different routes of sensitization: oral (CT adjuvant), skin, i.p. and last but not least oral but than with AGE-BSA as adjuvant. In addition, it studies ex vivo murine DC activation by AGE and AGE induced T-cell activation in human PBMC. This is brought into broader context, studying barrier disruptive effects of AGE diets and bacterial dysbiosis induced by the diet. The paper is well written and many different techniques were used to generate the results.

Yet also some critical issues arose when reviewing this paper. This is with regards to data analyses, data interpretation, statistical analyses and experimental set up. In addition, the discussion of the paper mainly discusses fairly lengthy the AGE effect on barrier, microbiome and possible contribution of RAGE and TLR4. It is unbalanced in this sense, since the food allergy models and immunological outcome as such are only limited discussed. Furthermore, when looking into the real proof that indeed the allergic response to ovalbumin (thus ovalbumin specific increase in symptoms, ova-IgE, cytokine release upon ex vivo OVA specific restimulation, ova-induced mast cell degranulation) this is either not significant or not measured in the oral ova-CT model and the skin model. Only the BSA-AGE-OVA model does this at the level of the allergy score, drop in temperature and ova-IgE (and ova-IgG1).

Major points of concern:

1) The database analyses discriminates between the relation food allergy or asthma or wheezing and AGE intake. There is correction for confounding based on age, gender, education level and BMI. However, no correction was done for dietary quality. For example fiber and anti-oxidant intake (fruit and vegetables) are associated with strong reduction in asthma risk. The link between AGE and asthma may therefore be not valid. The same would apply for food allergy, but more importantly in the cohort food allergy was not doctor reported but self diagnosed. In that perspective it is very hard to really pinpoint food allergy since it may also be other type of food related responses such as food intolerances that can be mistakenly noted as food allergy. Furthermore in the 'food allergy' group the RR is only increased in Q4 (no dose-response). What does this mean, this was not further discussed.

2) Statistical analyses: statistical analyses often is done by T-test, however this does not correct for multiple comparisons. Two way or one way anova (or kruskal wallis) with post hoc testing should be performed depending on the data set (if more than 2 groups are being compared within one analyses).

3) In Figure 2 it is shown that the HAGE diet itself enhances ovalbumin specific IgE and IgG1, how is this possible when HAGE does not contain ovalbumin? Can there be cross reactivity of epitopes in HAGE with ovalbumin? Please discuss.

4) The allergy score tends to increase in HAGE diet compared to control diet fed OVA allergic mice (figure 2), but is this through? Since OVA control mice have n=6 instead of n=8 (did two mice die for example from anaphylaxis?), it is unclear why data are missing (also at other occasions). In the humoral response there is no clear indication that HAGE increases

OVA sensitization. So from these data one can not conclude that allergic sensitization of or allergic symptoms are increased by the HAGE diet.

5) The mast cell counts are a bit difficult to appreciate based on histology only, allergen challenge should trigger release, serum mast cell mediators could be measured such as mucosal mast cell protease 1 (mmcp-1) in serum.

6) The lamina propria analyses of T-cells is interesting, but this is also not allergen specific, but generic T-cell skewing is shown. Allergen specific responses could be analyzed by ex vivo re-stimulation of LPMC (or MLN) with ovalbumin.

7) T-cell cytokines are plotted in figure 2j, but the data underlying this dataset is lacking.

8) Supplementary figure S5 which shows results for skin sensitization misses HAGE control group. Also in this model there are no indications that the allergen specific response to ovalbumin is further enhanced in HAGE fed mice compared to control fed mice. And the number n=8-26 is not explained in the methods. Results line 149 describe a trending severer ear thickness and 1.9 fold increase in total IgE. However, both are inadequate to allow the conclusion that ovalbumin food allergy / ova sensitization is increased upon feeding the AGE diet. Also the histology figure 5h is not supported by the relevant controls, nor quantified. In addition, Th2 increased as analyzed by cytof, but again this is not evidence for allergen specificity and not only T effector cells increase also regulatory T-cells increase (fig s6).

9) Histology pictures in figure 3f are difficult to appreciate since tissue architecture is not well kept. How to quantify this in a fair way?

10) Even though the authors indicate that TLR4 and RAGE 'signaling' contribute to type 2 driving cascade initiated by AGE, from the data it appears not that clear. AGE induces TLR4 and RAGE expression, as it activates NFkB, so it may be all part of a general inflammatory signature. It does not necessarily mean that AGE thus acts via TLR4 and RAGE signaling. In the knock out mice this is more clear. Different responses in the allergy model are modified by either TLR4-/- or RAGE-/- . However, it should be more clearly deciphered which receptor is contributing to what effect. And it should be discussed more precisely. In addition, there is the point that in the RAGE-/- mice basal alarmin expression and type 2 signature (IL-33 (tendency for TSLP), GATA3) is increased. This may mean that RAGE is even important in maintaining immune homeostasis, as it indicates immune activation in absence of RAGE. The question around the complex interplay between TLR4 and RAGE can only be hypothesized since no double knock out was used.

11) BMDC experiments with fecal water of HAGE fed mice does not have control fecal water of control fed mice. Thus it is not sure if AGE as caused this response.

Minor:

Title: the paper and abstract mainly indicate AGE (as Th2 adjuvant) induced type 2 skewing at the T cell level in the in vivo models. The title concerns innate signaling, apart from some analyses of alarmins in tissue and ex vivo DC activation the data do not further look into "innate signaling". Suggestion would be to adapt the title.

Introduction:

Please more clearly explain which procedures, heating temperature/ pressing/ additives that lead to AGE and explain a bit about different types of AGE and how these are measured in foods.

Please explain about food allergens, ovalbumin is not a random food allergen; hen's egg allergy is in the top 3 of major food allergic and ovalbumin is a strong allergen. Please explain why you have chosen ovalbumin as allergen in your models.

Please introduce the underlying immune pathways, how would AGE be able to drive type 2 polarization in gut and skin, since readers may be unaware of this.

Methods:

Please shortly describe (some examples) which type of foods are labeled as AGE containing.

What was the age of the mice? Sometimes n=5 was used for the studies some-times n=8 (and other numbers), please clearly explain in the methods the groups size per experiment.

What is the composition of the diet? Line 441: diet D12451H, line 443 D12450H?. Please refer to figure S2 showing quantification of AGE.

In the CT-OVA model mice receive many oral gavages with CT, this can be harmful for the mice? Did the authors observe any drop out? What is considered the humane end point in these studies please indicate in the methods.

How were the allergic symptoms scored?

Please explain missing values in data-sets.

BSA-MGO (BSA-AGE) was prepared. Please refer to supplemental figure S10 for composition. Please introduce BSA-AGE in the introduction as one of the main AGE that can be found in human(diet).

Line 476: indicate at which day permeability was measured and please refer to figure 3a.

Line 484: which commercial kits?

Line 488: "glycated protein" in stead of "protein"?

In the murine ovalbumin models the MLN re-stimulation was performed using PMA/ionomycin. In line 503 it says ex vivo activated cells. However, to study allergen specific responses this should be ovalbumin restimulation.

Line 517, supplemental table 2 is lacking.

Line 558, how were histological sections analyzed in a blinded way and how to control for differences in tissue morphology/architecture?

Line 591, 4 healthy volunteers (2 males...)?

Line 602, IFN in stead of INF

Line 610, please remove Nonparametric

Figure 6c, please remove the word 'concentration' from the y-axis

Supplementary figure S2 and S10 please remove statistical result for n=2. Statistics can be done from n=3 independent analyses onwards.

Supplementary figure S3 and in general: please remove the word 'irrelevant' before allergen OVA, maybe adapt it into model allergen OVA. It may be that the responses found are OVA related and not apply for all allergens. This cannot be told from these studies.

Line 119, BALB/c

Line 190, principal components analysis

Line 260, exist

Line 381, lamina

Reviewer #2

(Remarks to the Author)

This review starts with an epidemiological analysis of a North American Nutritional Survey and makes a case for ultra processed foods as a proxy for high dietary AGEs as being a risk factor for allergy. This adds to the literature. It sets the framework for the paper, but is not that novel in itself. There have been 3 more data-sets of nutritional surveys published since. Why were these not used or incorporated?

What is noteworthy is the series of in-vivo experiments that explore well recognised factors in food allergy such as microbiome and epithelial junctions and dAGEs ties this together.

The work supports the claims. The scientific tools and methods used are robust and appropriate. No claims are made outside the evidence. There is enough detail in the paper and supplementary material for replication of studies. This collective data should reach a plenary session in our field at a major US or European Meeting.

There are a few minimal grammar errors but generally this is well written.

I have a series of comments

Line 39

The Th2-stimulating effect of dAGEs involving RAGE-TLR4 crosstalk was finally validated in human peripheral immune cells

I would delate the word finally. You could add "Furthermore"... at the start of the sentence

Line 52

It would be strengthened by discussing that emigrants to western countries acquire the risk of food allergy and inhalant allergies when they move in the first 7 years of life or are born there. There are references particularly for the first 7 years for asthma – migrants to Canada and studies in Australia have shown higher rates of FA in first generation immigrants from Asia.

Line 61

Dietary AGEs (dAGEs) are a group of heterogeneous compounds formed between reducing sugars and the amino groups of proteins/peptides/free amino acids in food matrices via the non-enzymatic reaction
There are multiple non-enzymatic reactions and pathways to create dAGES

Line 159

"Additionally, a higher frequency of TLR4+ and RAGE+ cells in CD45+ cells was also noted in HAGE-groups, indicative of heightened pro161 inflammatory responses in lymphocytes under a high-AGE diet"

Do you mean there was higher TLR4 and RAGE expression on CD45+ cells?

Line 170

Increased diamine oxidase (DAO)

DAO degrades histamine, and this may be induced to cope with either increased histamine from allergies responses or

histamine producing microbiota

Line 260

can still be observed in RAGE 259 -/- mice

change "can" to "were". The sentence is in the past tense. English is a difficult language at times

Comments

E coli are slow growing species and make AGEs to poison bifidobacteria and lactobacilli which are faster growing. Their enzyme glyoxalase is mentioned in the manuscript, but this is pH dependent and is dramatically reduced if pH is increased (by antacids). Antacids are often used to create animal models of food allergy.

dAGES increase the longer food is stored. They form between sugars and proteins or lipids.

AGEs will also form with higher blood sugar levels. These will be endogenous formed AGEs with human lipids and proteins as all as AGEs made by microbiota. We should think of a total AGE pool. The western diet is high in sugar and all of this

We discussed this in:

Smith PK, Venter C, O'Mahony L, Canani RB, Lesslar OJL. Do advanced glycation end products contribute to food allergy? Front Allergy. 2023 Apr 4;4:1148181. doi: 10.3389/falgy.2023.1148181. PMID: 37081999; PMCID: PMC10111965.

We discussed the convergence of RAGE and TLR4 in the above paper - I am not seeking self reference, but this convergence of pathways has been described. It is pleasing to see it shown in animal models of food allergy

It is also important to recognise that Vitamin D – a strong epidemiological and in-vitro factor in food allergy has several intersections with RAGE and TLR4. Again, reviewed in the above paper.

I think the graphics and figures explain a lot of well conducted and thought-out experiments.

As a closing comment or even at the start of discussion, it is worth mentioning that AGEs appear to have an important role in multiple health disease and probably represent one of the most modifiable factors we have at our disposal.

I used AI to help me summarize a series of these (and I checked validity of each reference) .

Advanced Glycation End Products (AGEs) are proteins or lipids that become glycated after exposure to sugars. They accumulate in various tissues and have been implicated in the pathogenesis of numerous diseases, especially those related to aging, diabetes, and oxidative stress. Below is a list of diseases associated with high levels of AGEs, along with corresponding PubMed references.

1. Diabetes Mellitus and Diabetic Complications

High blood glucose levels in diabetes lead to increased formation of AGEs, contributing to various complications.

• Reference:

o Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005 Jun;54(6):1615-25. doi: 10.2337/diabetes.54.6.1615. PMID: 15919781.

2. Cardiovascular Disease

AGEs contribute to endothelial dysfunction and atherosclerosis, increasing the risk of cardiovascular events.

• Reference:

o Ott C, Jacobs K, Haucke E, et al. Role of advanced glycation end products in cellular signaling. Redox Biol. 2014 Jan;2:411-29. doi: 10.1016/j.redox.2013.12.016. PMID: 24624331; PMCID: PMC3939615.

3. Chronic Kidney Disease

Impaired renal function leads to decreased clearance of AGEs, promoting further kidney damage.

• Reference:

Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013 Jan;93(1):137-88. doi: 10.1152/physrev.00045.2011. PMID: 23303908.

4. Alzheimer's Disease and Neurodegenerative Disorders

AGEs accumulate in neural tissue, contributing to oxidative stress and neurodegeneration.

• Reference:

Li J, Liu D, Sun L, Lu Y, Zhang Z. Advanced glycation end products and neurodegenerative diseases: mechanisms and perspective. J Neurol Sci. 2012 Jun 15;317(1-2):1-5. doi: 10.1016/j.jns.2012.02.018. Epub 2012 Mar 11. PMID: 22410257.

5. Osteoporosis

AGEs affect bone quality by altering collagen properties, leading to decreased bone strength.

• Reference:

Ge W, Jie J, Yao J, Li W, Cheng Y, Lu W. Advanced glycation end products promote osteoporosis by inducing ferroptosis in osteoblasts. Mol Med Rep. 2022 Apr;25(4):140. doi: 10.3892/mmr.2022.12656. Epub 2022 Feb 25. PMID: 35211757; PMCID: PMC8908347.

6. Rheumatoid Arthritis

AGEs promote inflammation and joint destruction in rheumatoid arthritis patients.

• Reference:

Delrue C, Speeckaert R, Delanghe JR, Speeckaert MM. The Potential Influence of Advanced Glycation End Products and (s)RAGE in Rheumatic Diseases. Int J Mol Sci. 2023 Feb 2;24(3):2894. doi: 10.3390/ijms24032894. PMID: 36769213;

PMCID: PMC9918052.

7. Skin Aging and Disorders

Accumulation of AGEs in the skin leads to reduced elasticity and other signs of aging.

• Reference:

Pageon H. Reaction of glycation and human skin: the effects on the skin and its components, reconstructed skin as a model. *Pathol Biol (Paris)*. 2010 Jun;58(3):226-31. doi: 10.1016/j.patbio.2009.09.009. Epub 2009 Nov 5. PMID: 19896301.

8. Non-Alcoholic Fatty Liver Disease (NAFLD)

AGEs may exacerbate liver inflammation and fibrosis in NAFLD.

• Reference:

Gaens KH, Niessen PM, Rensen SS, Buurman WA, Greve JW, Driessen A, Wolfs MG, Hofker MH, Bloemen JG, Dejong CH, Stehouwer CD, Schalkwijk CG. Endogenous formation of Nε-(carboxymethyl)lysine is increased in fatty livers and induces inflammatory markers in an in vitro model of hepatic steatosis. *J Hepatol*. 2012 Mar;56(3):647-55. doi: 10.1016/j.jhep.2011.07.028. Epub 2011 Sep 10. PMID: 21907687.

9. Polycystic Ovary Syndrome (PCOS)

Increased AGEs may influence insulin resistance and hormonal imbalances in PCOS.

• Reference:

Diamanti-Kandarakis E, Piperi C, Kalofoutis A, Creatsas G. Increased levels of serum advanced glycation end-products in women with polycystic ovary syndrome. *Clin Endocrinol (Oxf)*. 2005 Jan;62(1):37-43. doi: 10.1111/j.1365-2265.2004.02170.x. PMID: 15638868.

10. Cataracts

AGEs in lens proteins contribute to lens opacity and cataract formation.

• Reference:

Franke S, Dawczynski J, Strobel J, Niwa T, Stahl P, Stein G. Increased levels of advanced glycation end products in human cataractous lenses. *J Cataract Refract Surg*. 2003 May;29(5):998-1004. doi: 10.1016/s0886-3350(02)01841-2. PMID: 12781289.

11. Erectile Dysfunction

AGE-induced vascular damage can lead to erectile dysfunction.

• Reference:

Cellek, S., Cameron, N., Cotter, M. et al. Pathophysiology of diabetic erectile dysfunction: potential contribution of vasa nervorum and advanced glycation endproducts. *Int J Impot Res* 25, 1–6 (2013). <https://doi.org/10.1038/ijir.2012.30>

12. Inflammatory Bowel Disease (IBD)

AGEs may play a role in the inflammatory processes of IBD.- CD but not UC.

• Reference:

Jiang F, Yang W, Cao Y, Cao X, Zhang Y, Yao L, Cao Q. The contribution of dietary advanced glycation end-products and genetic risk in the development of inflammatory bowel disease: a prospective cohort study. *Aliment Pharmacol Ther*. 2024 Oct;60(8):1075-1086. doi: 10.1111/apt.18218. Epub 2024 Aug 23. PMID: 39177057.

13. Cancer

AGEs can may increase risk of cancer and promote tumor growth and metastasis through various mechanisms.

• Reference:

Turner DP. Advanced glycation end-products: a biological consequence of lifestyle contributing to cancer disparity. *Cancer Res*. 2015 May 15;75(10):1925-9. doi: 10.1158/0008-5472.CAN-15-0169. Epub 2015 Apr 28. PMID: 25920350; PMCID: PMC4433613.

Muthyalaiyah YS, Jonnalagadda B, John CM, Arockiasamy S. Impact of Advanced Glycation End products (AGEs) and its receptor (RAGE) on cancer metabolic signaling pathways and its progression. *Glycoconj J*. 2021 Dec;38(6):717-734. doi: 10.1007/s10719-021-10031-x. Epub 2022 Jan 22. PMID: 35064413.

14. Peripheral Artery Disease

AGEs contribute to vascular stiffness and impaired blood flow in peripheral arteries.

• Reference:

Garg PK, Biggs ML, Barzilay J, Djousse L, Hirsch C, Ix JH, Kizer JR, Tracy RP, Newman AB, Siscovick DS, Mukamal KJ. Advanced glycation end product carboxymethyl-lysine and risk of incident peripheral artery disease in older adults: The Cardiovascular Health Study. *Diab Vasc Dis Res*. 2019 Sep;16(5):483-485. doi: 10.1177/1479164119847481. Epub 2019 May 8. PMID: 31064218; PMCID: PMC6697602.

15. Chronic Obstructive Pulmonary Disease (COPD)

AGEs may exacerbate inflammation and tissue damage in COPD.

• Reference:

Hoonhorst SJ, Lo Tam Loi AT, Pouwels SD, Faiz A, Telenga ED, van den Berge M, Koenderman L, Lammers JW, Boezen HM, van Oosterhout AJ, Lodewijk ME, Timens W, Postma DS, Ten Hacken NH. Advanced glycation endproducts and their receptor in different body compartments in COPD. *Respir Res*. 2016 Apr 26;17:46. doi: 10.1186/s12931-016-0363-2. PMID: 27117828; PMCID: PMC4847335.

(Remarks to the Author)

1) General comment

In their manuscript entitled: "Diet rich in advanced glycation end-products misdirected intestinal mucosa immunity to promote food allergy through innate signaling" Zhang et al. establish a link between advanced glycation end-product (AGE) from ultra-processed food (UPF) and the onset and development of allergic diseases. First, using an epidemiologic investigation based on a survey about diet and allergy, they establish a correlation between AGE containing UPS consumption and allergic risk. Then, using different mouse models of food allergy, authors demonstrate a link between chronic AGE consumption and inflammation highlighting possible molecular pathways involved. Authors raise an important question to unravel the important rise of allergic diseases the last decade linking recent hypothesis about barrier degradation and allergy development. Authors show an important amount of data to support their conclusion, however authors should more moderate to not risk to overstate their conclusion. Therefore, some modification may help to improve manuscript impact.

1) Specific major comment

In the first part of their manuscript, authors used a questionnaire involving a large amount of person to correlate AGE consumption and allergic diseases. In the discussion section, authors should also consider the impact of diet from people with less UPF consumption as a protective effect from allergic disease development. In fact, it has been demonstrated in human and in animal model that fiber or anti-oxidant consumption are protective factor and food allergy or asthma exacerbation development and severity (Moro et al 2006 ; Ziegler et al., 2008 ; Braithwaite et al 2014 ; Protudjer et al., 2012). Within the same line, as allergic diseases are based on self-report, authors should be more careful in their conclusion (for instance : L104: "asthma" should be replaced by self-report asthma or asthma symptoms feeling).

Then, in a second part of the manuscript, authors use orally and percutaneous model of food allergy to demonstrate that AGE exposure increase allergy severity and development. Here again, conclusion might be overstated. In fact, authors clearly demonstrate an inflammatory effect of AGE exposure regarding IgG1, Total IgE, mast cell in colon, CD8 infiltrate, Th2 cells and Treg cells. However, increase of allergic diseases severity is less clear as allergy score (based on subjective parameters such as scratching or behavior) is not increase comparing SD FA to HAGE FA. Similarly, specific IgE, Treg and CD4 T cells remains similar between SD and HAGE groups in allergic conditions. Other allergic marker based on allergic symptoms manifestation measurement such as histamine, mMCP1, gut permeability may help to reinforce effect of AGE in allergic conditions. Nevertheless, modification has to be made in results description such as L138, L144 and L163 where HAGE mice seems to be in a low inflammatory condition more than in allergic exacerbation both in oral and percutaneous model.

In a third part of their manuscript, authors demonstrate the adjuvant effect of AGE consumption. Using mouse model of adjuvant free orally sensitized mice, authors demonstrate an increase of global gut permeability after 12 weeks of AGE diet. However, they showed DAO and Lactic acid (Figure 3d and e) only in HAGE-OVA fed mice, also it is difficult to measure impact of OVA bound to AGE without depicting AGE fed mice on the same graph (as for TJ and TLR expression). Similarly, for microbial diversity measurement (Figure 3i and j), mice fed with AGE, SD, SD-OVA and HAGE-OVA at week 16 should be depicted. Then, using BSA-AGE as an adjuvant in orally sensitized food allergy to OVA model, authors demonstrate FA development in AGE-OVA sensitized mice but not in OVA sensitized mice based on a slight increase in body temperature drop, sIgE level and allergy score. However, as AGE adjuvant effect seems restricted to orally sensitization, it would be of interest to compare adjuvant effect of AGE with common adjuvant known for oral sensitization such as cholera toxin deciphering the potential adjuvant effect of AGE to a global inflammatory effect as suggested by the increase of Th1 cells (Figure 4g).

Finally, authors explore molecular pathways involves in AGE adjuvant effect using TLR4 and RAGE KO mice and show that TLR4 KO as well as RAGE KO mice do not develop allergic reaction as suggest by the allergy score, body temperature drop and specific IgE (Figure 5a-e). As no allergic disease is observed in AGE as well as AGE-OVA experimental condition neither in TLR4 or RAGE KO mice, it is unclear whether TLR4 and/or RAGE is specifically involved in the allergic process. It would have been interested to include OVA sensitized and challenged mice in TLR4 and RAGE KO mice to reinforce the role of AGE not only in inflammation but in the allergic process. Authors should discuss the high level of specific IgE observed in RAGE KO mice (Figure 5d). Similarly, PBMC should be exposed to an allergen induce changes in bacterial metabolite such as OVA to measure the impact of AGE exposure impact on bacterial metabolites in the allergic context.

2) Specific minor comment

- L120: Authors should add the starting point of the 12 weeks AGE exposure as Figure 2a show a negative starting point meaning pregnant mice were exposed? Similarly age of mice should mention in the material and methods section.
- Figure 4b should be cited before figure 4c. Text might modify or panel order in Figure4.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the rebuttal to my comments and the revised manuscript, authors carefully addressed all issues raised. In particular the addition of the OVA specific T-cell analyses using tetramers is interesting and convincing that indeed a systemic ova specific Th2-cell response is generated in the AGE + OVA sensitization model.

Also intestinal mast cell protease expression was assessed. With regard to this staining, it is a bit hard to appreciate if indeed this shows enhanced mast cell activation and degranulation (quality is not very sharp). As the immunohistochemistry

pictures do not show (degranulating) mast cells. The expression levels of MCT and (possibly) MCC (but MCC was also already high in the controls which would not be expected) do seem to be affected. It is unclear at which enlargement these pictures were taken. Hence these additions are interesting but care should be taken in making strong conclusions.

Of note:

- Fig 4 y-axis states total IgEn should this be total IgE?
- Fig S3 y-as should probably state colon in stead of jejunum

Reviewer #2

(Remarks to the Author)

I am delighted with this paper in its current form. I think the diversity of reviewer comments, extra experiments and further clarifications / edits have made this a solid paper, worthy of the journal. The paper expresses their findings and limitations. I expect it will be heavily cited as a key paper in the mechanisms of food allergy. I support acceptance in the current form.

Reviewer #3

(Remarks to the Author)

Authors reply to all comment improve the manuscript. In particular, they moderate their conclusion regarding allergic diseases development and focusing their conclusion on inflammation and inflammatory process rather than of allergy. No further comment.

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Detailed Response to the Editor and Reviewers

Reviewer #1:

This manuscript deals with the dietary contamination of AGE products that are introduced during food processing and the risk on developing food allergy as AGE may act as and type 2 skewing adjuvant. This topic is very relevant and many of the data shown in this manuscript and the supplementary figures are of interest. The paper shows analyses of an observational study, the effects of an AGE rich diet in three different ovalbumin induced murine models. These contain different routes of sensitization: oral (CT adjuvant), skin, i.p. and last but not least oral but than with AGE-BSA as adjuvant. In addition, it studies ex vivo murine DC activation by AGE and AGE induced T-cell activation in human PBMC. This is brought into broader context, studying barrier disruptive effects of AGE diets and bacterial dysbiosis induced by the diet. The paper is well written and many different techniques were used to generate the results.

[1]. Yet also some critical issues arose when reviewing this paper. This is with regards to data analyses, data interpretation, statistical analyses and experimental set up. In addition, the discussion of the paper mainly discusses fairly lengthy the AGE effect on barrier, microbiome and possible contribution of RAGE and TLR4. It is unbalanced in this sense, since the food allergy models and immunological outcome as such are only limited discussed. Furthermore, when looking into the real proof that indeed the allergic response to ovalbumin (thus ovalbumin specific increase in symptoms, ova-IgE, cytokine release upon ex vivo OVA specific restimulation, ova-induced mast cell degranulation) this is either not significant or not measured in the oral ova-CT model and the skin model. Only the BSA-AGE-OVA model does this at the level of the allergy score, drop in temperature and ova-IgE (and ova-IgG1).

Author's Response: We sincerely thank the reviewer for their constructive feedback. In the revised manuscript, we have strengthened the discussion of food allergy models and immunological outcomes to better balance the focus on AGE mechanisms (*lines 336-354*). Additionally, while the long-term exposure model showed non-allergen-specific responses rather than elevated OVA sIgE, it is evidence that high-AGE diets exacerbate allergic inflammation, as supported by increased mast cell infiltration and Th2/Treg imbalance in the intestine. To further substantiate these findings, we conducted additional experiments, including:

① *Immunohistochemical staining of mast cell tryptase and chymase in colon tissues*: In the orally sensitized model, staining of mast cell-associated proteases revealed that high-AGE diets significantly upregulated the expression of MCT and MCC in sensitized mice, indicating more severe mast cell degranulation and activation (*new data in Supplementary Fig. S3*).

② Tetramer analyses of OVA-specific T-cell responses: In the BSA-AGE model, we performed tetramer analysis to assess OVA-specific T-cell responses. Splenocytes from sensitized mice were stimulated and stained with an IAb tetramer specific for the OVA₃₂₃₋₃₃₉ epitope. Results demonstrated that only the AGE+OVA group, but not the OVA group, exhibited a significantly higher frequency of CD4⁺ tetramer-positive cells, confirming that AGEs exposure amplifies OVA-specific Th2 responses. This directly supported AGEs' role in enhancing antigen-driven Th2 polarization (new data in **Supplementary Fig. S10**).

We hope these revisions adequately address the concerns raised and further clarify the mechanisms by which AGEs contribute to food allergic pathogenesis.

- [2]. The database analyses discriminate between the relation food allergy or asthma or wheezing and AGE intake. There is correction for confounding based on age, gender, education level and BMI. However, no correction was done for dietary quality. For example, fiber and anti-oxidant intake (fruit and vegetables) are associated with strong reduction in asthma risk. The link between AGE and asthma may therefore be not valid. The same would apply for food allergy, but more importantly in the cohort food allergy was not doctor reported but self diagnosed. In that perspective it is very hard to really pinpoint food allergy since it may also be other type of food related responses such as food intolerances that can be mistakenly noted as food allergy. Furthermore in the 'food allergy' group the RR is only increased in Q4 (no dose-response). What does this mean, this was not further discussed.

Author's Response: We thank the reviewer for these constructive comments. As suggested, we have now incorporated dietary antioxidant and fiber intake as additional covariates in our statistical models. After adjustment, the association between high-AGE UPFs intake and allergic diseases remained significant, suggesting that this relationship is independent of dietary quality (**Fig. 1, Table S1, and Fig. S1**).

We also acknowledge the limitations of self-reported allergic data and replace terms like "food allergy" with "self-reported food allergy" to avoid overstate our results (**lines 111-124**).

Regarding the increased food allergy risk only in the highest quartile (Q4), potential explanations may include threshold effects, population heterogeneity or compounding factors, and this has been discussed in the revised Results section (**lines 121-123**). We hope these revisions address the reviewer's concerns and strengthen the manuscript.

- [3]. Statistical analyses: statistical analyses often is done by T-test, however this does not correct for multiple comparisons. Two way or one way anova (or kruskal wallis) with post hoc testing should

be performed depending on the data set (if more than 2 groups are being compared within one analyses).

Author's Response: We appreciate the reviewer's valuable suggestion. We have re-analyzed all relevant data (*i.e.*, comparisons between three and more groups) using one-way ANOVA or Kruskal-Wallis test followed by appropriate post hoc tests, as recommended. All figures, statistical annotations, and result interpretations have been updated accordingly. For those figures that statistical comparison was performed between two groups, Student's *t* or Mann-Whitney U tests were retained. We believe these corrections have significantly strengthened the statistical rigor of our study.

#Sentences added or revised:

(**Lines 698-704**) Comparisons between two groups were conducted using Student's *t*-tests for normally distributed data or Mann-Whitney U tests for non-normally distributed data. For comparisons involving three or more groups, one-way ANOVA with Tukey's multiple comparison test was applied to normally distributed data, while Kruskal–Wallis test with Dunn–Bonferroni post hoc analysis was used for non-normally distributed data.

- [4]. In Figure 2 it is shown that the HAGE diet itself enhances ovalbumin specific IgE and IgG1, how is this possible when HAGE does not contain ovalbumin? Can there be cross reactivity of epitopes in HAGE with ovalbumin? Please discuss.

Author's Response: We sincerely appreciate the reviewer's insightful comments. It should be pointed out that after 12-week HAGE feeding, the mice received four times of high-dose ovalbumin (OVA) gavage without adjuvant. This repeated OVA exposure, in the context of the HAGE diet, led to the observed increase in OVA-specific antibodies. The HAGE diet likely exerts an adjuvant-like effect, that enhances the immune response to subsequently administered antigens like OVA. These findings also inspired our subsequent development of the adjuvant-free experimental model. We have clarified this important point in the revised manuscript (**lines 145-152**).

#Sentences added or revised:

(**Lines 145-152**) Intriguingly, feeding mice with HAGE alone without allergen sensitization but with only high-dose OVA challenges was sufficient to elicit both total and antigen-specific IgG1 responses (HAGE vs. SD; Fig. 2d,e). The expression level of mast cell proteases in the colon tissue was also higher in mice fed HAGE vs. SD control (HAGE vs. SD; Supplementary Fig. 3c). This suggests that prolonged consumption of a high-AGE diet induced allergic inflammatory responses, even after a few doses of antigen exposure. The HAGE diet likely exerts an adjuvant-

like effect, priming the immune system and enhancing its responsiveness to subsequently encountered allergens such as OVA.

- [5]. The allergy score tends to increase in HAGE diet compared to control diet fed OVA allergic mice (figure 2), but is this through? Since OVA control mice have n=6 instead of n=8 (did two mice die for example from anaphylaxis?), it is unclear why data are missing (also at other occasions). In the humoral response there is no clear indication that HAGE increases OVA sensitization. So from these data one cannot conclude that allergic sensitization of or allergic symptoms are increased by the HAGE diet.

Author's Response: We sincerely thank the reviewer's insightful comments. Regarding the missing data, we have clarified in the revised manuscript and source data file that the discrepancies in sample sizes are due to either variations in group sizes or the exclusion of extreme outliers based on predefined statistical criteria. No mice died from anaphylaxis or other experimental procedures.

We agree with the reviewer that, in the long-term exposure model, HAGE did not enhance allergen-specific antibody responses. However, it's robust that HAGE significantly exacerbated allergic inflammation, as evidenced by increased mast cell degranulation and elevated Th2 cell proportions. Even in non-allergic mice, HAGEs induced systemic inflammation, as shown by elevated total IgE levels, increased mast cell degranulation, and Th2/Treg imbalance. To further address the role of HAGE in allergen-specific responses, we conducted additional experiments in the BSA-AGE model. By staining splenocytes with an IAb tetramer specific for the OVA₃₂₃₋₃₃₉ epitope, we demonstrated that AGEs exposure amplifies OVA-specific Th2 responses (**new data in Supplementary Fig. S10**). We hope these revisions and additional data address the reviewer's concerns and strengthen the conclusions of our study.

- [6]. The mast cell counts are a bit difficult to appreciate based on histology only, allergen challenge should trigger release, serum mast cell mediators could be measured such as mucosal mast cell protease 1 (mmcp-1) in serum.

Author's Response: We appreciated the reviewer's constructive suggestions. To further address the effect of AGEs on allergen-induced mast degranulation, we have performed immunohistochemical staining of mast cell tryptase (MCT) and chymase (MCC) in colon tissues from the orally sensitized model. The results revealed that high-AGE diets significantly increased the expression of MCT and MCC in sensitized mice, providing direct evidence of enhanced mast cell activation and degranulation in the intestinal tissue (**new data in Supplementary Fig.**

S3). We believe these findings address the reviewer's concerns and significantly enhance the robustness of our manuscript.

- [7]. The lamina propria analyses of T-cells is interesting, but this is also not allergen specific, but generic T-cell skewing is shown. Allergen specific responses could be analyzed by ex vivo re-stimulation of LPMC (or MLN) with ovalbumin.

Author's Response: We appreciate the reviewers valuable suggestion. To assess this, we performed tetramer analysis in the BSA-AGE model to assess OVA-specific T-cell responses. Splenocytes from mice with sensitized with OVA, with or without AGE, were stimulated and stained with an IAb tetramer specific for the OVA₃₂₃₋₃₃₉ epitope. Results demonstrated that only the AGE+OVA group, but not the OVA group, exhibited a significantly higher frequency of CD4⁺ tetramer-positive cells, confirming that AGEs exposure enhances OVA-specific Th2 responses (*new data in Supplementary Fig. S10*). We hope these revisions and additional data address the reviewer's concerns and strengthen the conclusions of our study.

- [8]. T-cell cytokines are plotted in figure 2j, but the data underlying this dataset is lacking.

Author's Response: We thank the reviewer's attention to detail. The dataset underlying the T-cell cytokine analysis in **Figure 2j** has been provided in the **Source Data file**, specifically in the sheet labeled *Fig. 2j*.

- [9]. Supplementary figure S5 which shows results for skin sensitization misses HAGE control group. Also in this model there are no indications that the allergen specific response to ovalbumin is further enhanced in HAGE fed mice compared to control fed mice. And the number n=8-26 is not explained in the methods. Results line 149 describe a trending severer ear thickness and 1.9 fold increase in total IgE. However, both are inadequate to allow the conclusion that ovalbumin food allergy / ova sensitization is increased upon feeding the AGE diet. Also the histology figure 5h is not supported by the relevant controls, nor quantified. In addition, Th2 increased as analyzed by cytof, but again this is not evidence for allergen specificity and not only T effector cells increase also regulatory T-cells increase (fig s6).

Author's Response: We sincerely thank the reviewer for their insightful comments.

① *Absence of HAGE control group in the skin sensitization model:* We understand the reviewer's concern about the absence of an HAGE control group (without OVA sensitization) in the skin sensitization model. Here we focused on comparing the effects of HAGE vs. SD in the context of OVA sensitization to directly evaluate how HAGE exacerbates allergic responses. Importantly, the other long-term dietary intervention model (oral sensitization) included a full set of controls (SD, SD-OVA, HAGE, and HAGE-OVA), which consistently demonstrated that HAGE

exacerbates allergic inflammation. We believe the results from both models collectively support our conclusions.

② *OVA-specific sensitization and inflammatory responses:*

We agree that the trending increase in ear thickness and the 1.9-fold rise in total IgE levels alone are insufficient to conclusively state that HAGE enhances OVA-specific sensitization. To address this, we have revised the text to avoid overstating conclusions about "allergy susceptibility" and instead focus on the exacerbation of allergic inflammatory responses (*lines 140-177*). Pathological analysis corroborated these findings, revealing more severe intestinal tissue injury (e.g., disrupted connections and autolysis of jejunal villi) in HAGE-sensitized mice compared to controls.

To further confirm that the inflammatory responses are allergen-specific, we analyzed OVA-specific T-cell responses in the BSA-AGE model. Splenocytes were stimulated and stained with an I-Ab tetramer specific for the OVA₃₂₃₋₃₃₉ epitope. Results demonstrated that OVA exposure in the presence of AGE significantly enhances OVA-specific Th2 responses, supporting BSA-AGE acts as an adjuvant to promote allergen-specific Th2 immunity. These data are now included in *Fig. S10* and Results section (*lines 232-237*) to support our conclusions.

We hope these revisions address the reviewer's concerns and strengthen the manuscript.

[10]. Histology pictures in figure 3f are difficult to appreciate since tissue architecture is not well kept. How to quantify this in a fair way?

Author's Response: We sincerely thank the reviewer for this valuable comment. We have re-performed the immunohistochemical staining to ensure the preservation of complete tissue morphology (*updated Fig. 3f and S7*). Quantification of the average optical density was using ImageJ, a widely recognized and standardized software for image analysis.

[11]. Even though the authors indicate that TLR4 and RAGE 'signaling' contribute to type 2 driving cascade initiated by AGE, from the data it appears not that clear. AGE induces TLR4 and RAGE expression, as it activates NF- κ B, so it may be all part of a general inflammatory signature. It does not necessarily mean that AGE thus acts via TLR4 and RAGE signaling. In the knock out mice this is more clear. Different responses in the allergy model are modified by either TLR4^{-/-} or RAGE^{-/-}. However, it should be more clearly deciphered which receptor is contributing to what effect. And it should be discussed more precisely. In addition, there is the point that in the RAGE^{-/-} mice basal alarmin expression and type 2 signature (IL-33 (tendency for TSLP), GATA3) is increased. This may mean that RAGE is even important in maintaining immune homeostasis, as

it indicates immune activation in absence of RAGE. The question around the complex interplay between TLR4 and RAGE can only be hypothesized since no double knock out was used.

Author's Response: We thank the reviewer for raising these insightful comments. Below, we address their concerns:

① *Specific contributions of TLR4 and RAGE signaling:*

Our data from RAGE^{-/-} and TLR4^{-/-} mice demonstrate distinct roles for these receptors in dAGEs-mediated allergic inflammation. Specifically:

- **TLR4 signaling** drives early barrier disruption and localized inflammation, as evidenced by activation of MyD88, elevated IL-33 levels (a marker of epithelial damage), and a trend toward increased GATA3 expression in RAGE^{-/-} mice. However, TLR4 activation alone is insufficient to drive the full Th2 immune response required for allergic sensitization.

- **RAGE signaling** is critical for later-stage immune cell activation and allergen-specific responses. In RAGE^{-/-} mice, despite TLR4-mediated early inflammation (e.g., IL-33 and MyD88 activation), the absence of RAGE abolishes downstream antibody responses (e.g., OVA-specific IgE) and Th2 immune activation.

Overall, we postulated that TLR4 drives early events, while RAGE is essential for later immune activation. We have revised the discussion to more explicitly highlight these distinct contributions.

② *Increased basal alarmin and Th2 signature in RAGE^{-/-} mice:*

Thank you for pointing this out. This intriguing finding aligns with our observations that TLR4 and its adaptor molecule MyD88 are activated in RAGE^{-/-} mice, accompanied by elevated IL-33 levels and a trending increased GATA3 expression in the intestine. We propose that TLR4 signaling may contribute to early barrier disruption and localized inflammation, as IL-33 is a danger signal released from damaged epithelium, and MyD88/NF-κB is a classic pro-inflammatory pathway. However, TLR4 activation alone appears insufficient to drive the full Th2 immune response required for allergic sensitization, highlighting the important role of RAGE in later sensitization processes. This point has been discussed in the revised manuscript.

③ *Complex interplay between TLR4 and RAGE:*

We acknowledge the reviewer's point regarding the complex interplay between TLR4 and RAGE. While our study did not include double knockout mice, our data suggest that TLR4 and RAGE signaling may act synergistically to amplify dAGEs-induced allergic inflammation. For example, in RAGE^{-/-} mice, we observed upregulation of TLR4 and its adaptor molecule MyD88, indicating potential compensatory mechanisms. However, this TLR4 activation alone was insufficient to drive full allergic responses, underscoring the importance of RAGE in this process.

We have revised the discussion to more explicitly hypothesize the potential crosstalk between these receptors and its implications for food allergy pathogenesis. We have noted this as a study limitation, emphasizing the need for future studies using double KO and tissue- or cell-specific KO models to further dissect the crosstalk between RAGE and TLR4 signaling and its role in allergic sensitization.

#Sentences added or revised:

(**Lines 283-287**) As a consequence, TLR4^{-/-} mice were protected from disruptions in gut barrier integrity and Th2-biased pro-inflammatory responses in the intestine, whereas significant increases in IL-33 (a danger signal released from damaged epithelium) and a trend toward increased GATA3 (the key transcription factor for Th2 response) expression were still observed in RAGE^{-/-} mice.

(**Lines 425-431**) Interestingly, in RAGE-deficient mice, we observed up-regulation of TLR4 and its adaptor molecule MyD88, along with elevated IL-33 levels and a trending higher GATA3 expression in the intestine. However, activation of TLR4 alone is insufficient to drive the full Th2 immune response required for allergic sensitization. These findings suggest that TLR4 and RAGE signaling may contribute to distinct stages of dAGEs-induced allergic inflammation: TLR4 drives early barrier disruption and localized inflammation, while RAGE is essential for later-stage immune cell activation and allergen-specific responses.

(**Lines 446-450**) Another limitation is that we adopted conventional KO mouse instead of double KO mouse or tissue-/cell-specific KO models. Subsequent research employing targeted genetic manipulation would help to elucidate the precise role of RAGE-TLR4 crosstalk on specific cell types in manipulation of the intricate interplay between dAGEs and allergic sensitization.

[12].BMDC experiments with fecal water of HAGE fed mice does not have control fecal water of control fed mice. Thus it is not sure if AGE as caused this response.

Author's Response: We thank the reviewer for raising this important point. We have now included additional experiments using fecal supernatant from control-fed mice (FM-SD) as a control for the fecal supernatant from HAGE-fed mice (FM-HAGE). The results demonstrate that FM-HAGE, but not FM-SD, significantly enhanced the activation and cytokine production of PBMCs, confirming that the observed effects are indeed attributable to AGE-related metabolites in the fecal supernatant. These data have been added to **Fig. 6, Fig. S14**, and described in the revised Results section (**lines 310-318**). We believe these additional experiments address the reviewer's concern and strengthen our conclusions.

Minors:

- [2] **Title:** the paper and abstract mainly indicate AGE (as Th2 adjuvant) induced type 2 skewing at the T cell level in the in vivo models. The title concerns innate signaling, apart from some analyses of alarmins in tissue and ex vivo DC activation the data do not further look into 'innate signaling'. Suggestion would be to adapt the title.

Author's Response: We appreciate the reviewer's insightful comments regarding the title of our manuscript. We have revised the title to better align with the main conclusions of our work. The new title is: "***Dietary advanced glycation end-products promote food allergy by disrupting intestinal barrier and enhancing Th2 immunity***". This revised title more accurately captures the essence of our findings, which demonstrate that dietary AGEs promote food allergy primarily through disrupting the barrier function and inducing Th2-biased immune responses in the intestinal mucosa. We hope this change addresses the reviewer's concern and improves the clarity of our manuscript.

- [3] **Introduction:**

- Please more clearly explain which procedures, heating temperature/ pressing/ additives that lead to AGE and explain a bit about different types of AGE and how these are measured in foods.

Author's Response: We thank the reviewer for this important question. The details related to AGEs types and analysis has now been included (**lines 68-71**). We also added some examples of high-AGE foods to provide readers with a clearer understanding of their dietary sources.

- Please explain about food allergens, ovalbumin is not a random food allergen; hen's egg allergy is in the top 3 of major food allergic and ovalbumin is a strong allergen. Please explain why you have chosen ovalbumin as allergen in your models.

Author's Response: Thank you for the valuable comments. As noted, hen's egg allergy ranks among the most common food allergies globally, and ovalbumin—the dominant allergenic protein in egg white—is a well-characterized and potent food allergen. We selected ovalbumin as a model allergen to reflect its clinical prevalence and relevance to real-world food allergy challenges, ensuring translational relevance. This rationale is now clarified in the revised Introduction and Results section (**lines 47-48, 139**).

- Please introduce the underlying immune pathways, how would AGE be able to drive type 2 polarization in gut and skin, since readers may be unaware of this.

Author's Response: We appreciate the reviewer's valuable suggestion. While the direct link between AGEs and type 2 immunity remains unexplored in existing literature, emerging evidence suggests potential pathways through gut homeostasis disruption. Specifically, AGEs may promote type 2 polarization through the following pathways: 1) inducing epithelial barrier

dysfunction that enhance subsequent allergen sensitization; 2) triggering localized inflammation that primes Th2 responses; and 3) altering gut microbiota composition, which modulates immune polarization. These mechanisms have been discussed in the revised Introduction (*lines 79-84*).

[4] **Methods, Tables and Figures:**

- Please shortly describe (some examples) which type of foods are labeled as AGE containing.

Author's Response: We thank the reviewer for the valuable advice. Representative examples of high-AGE foods, such as grilled meats, fried products, and baked goods, have been mentioned in the revised introduction (*lines 68-69*).

- What was the age of the mice? Sometimes n=5 was used for the studies some-times n=8 (and other numbers), please clearly explain in the methods the groups size per experiment.

Author's Response: Thank you for pointing this out. All experiments were conducted using 4~6-week-old mice, and this has now been stated in the Methods (*line 494*). Regarding group size in different experiments, these have been consistently reported in the corresponding figure legends.

- What is the composition of the diet? Line 441: diet D12451H, line 443 D12450H?. Please refer to figure S2 showing quantification of AGE.

Author's Response: We apologize for this mistake. The name of the control diet is D12450H, and its ingredient information is now provided in *Table S2*.

- In the CT-OVA model mice receive many oral gavages with CT, this can be harmful for the mice? Did the authors observe any drop out? What is considered the humane end point in these studies please indicate in the methods.

Author's Response: We thank the reviewer for raising these important points. We confirm that CT was administered orally at a standard dose established as safe in food allergy models, with no observed adverse effects (e.g., diarrhea, weight loss) or dropouts attributable to CT throughout the sensitization period. Humane endpoints predefined per institutional guidelines (weight loss $\geq 20\%$, severe distress, or irreversible morbidity) were rigorously monitored. These details have been added to the Methods section (*lines 503-506*).

#Sentences added or revised:

(*Lines 503-506*) In particular, mice were monitored daily for signs of distress, including weight loss $\geq 20\%$, severe diarrhea, or irreversible morbidity, as predefined humane endpoints per institutional guidelines. No adverse effects attributed to adjuvants exposure were observed during the study.

- How were the allergic symptoms scored?

Author's Response: Thank you for the comments. We employed a standardized scoring system based on well-established criteria from previous reports. The detailed information is provided in **Table S3**.

- Please explain missing values in data-sets.

Author's Response: We appreciate the reviewer's attention to this detail. The missing data in our study primarily arise from two sources: (1) variations in group sizes due to differing numbers of mice across different groups, and (2) the exclusion of extreme outliers identified by statistical test. To ensure transparency, we have detailed the outlier removal process in the Statistical Methods section (**lines 702-704**) and have explicitly noted these cases in the relevant Source Data files where applicable.

#Sentences added or revised:

(**Lines 702-704**) Extreme outliers, defined as data points exceeding the 75th percentile plus 1.5 times the interquartile range (IQR) or falling below the 25th percentile minus 1.5 times the IQR, were excluded.

- BSA-MGO (BSA-AGE) was prepared. Please refer to supplemental figure S10 for composition. Please introduce BSA-AGE in the introduction as one of the main AGE that can be found in human(diet).

Author's Response: We thank the reviewer for this valuable suggestion. Dietary AGEs are diverse, arising from various proteins reacting with reducing sugars under heating conditions. Here, we selected BSA-MGO (BSA-AGE) as a representative model, due to the widespread use of BSA as a standard protein in biochemical studies. Its well-characterized properties and relevance to dietary protein modifications make it an ideal candidate for investigating AGE-related mechanisms. This rationale has been clarified in the revised Method and Results Sections (**line 225, 526**), as we believe the detailed description of BSA-AGE is more relevant to the experimental design and model selection than the Introduction.

- Line 476: indicate at which day permeability was measured and please refer to figure 3a.

Author's Response: Thank you for pointing this out. We have explicitly stated these time points in the revised manuscript for clarity (**lines 548-549**).

- Line 484: which commercial kits?

Author's Response: The information of the two commercial kits is supplied (**lines 555-556**).

- Line 488: 'glycated protein' instead of 'protein'?

Author's Response: Thank you for the advice. However, the word “protein” in this context represents total protein fraction in the diet sample, including those non-glycated and glycated.

- In the murine ovalbumin models the MLN re-stimulation was performed using PMA/ionomycin. In line 503 it says ex vivo activated cells. However, to study allergen specific responses this should be ovalbumin restimulation.

Author's Response: Thank you for the valuable comments. In the long-AGE exposure models, PMA/ionomycin stimulation was used to assess non-specific T cell differentiation across subsets (e.g., Th1/Th2 ratios) in MLN cells. To specifically evaluate OVA-specific responses, we conducted additional experiments in the adjuvant-free model by using an I-Ab tetramer specific for OVA epitope to stimulate mouse splenocytes. Results demonstrated that OVA exposure with AGE significantly enhanced allergen-specific Th2 responses. These data are now included in **Fig. S10** and Results section (**lines 232-237**) to support our conclusions.

- Line 517, supplemental table 2 is lacking.

Author's Response: We apologize for this mistake. Supplementary Table 2 is now provided as **Table S4** in the file “Supplementary Tables”.

- Line 558, how were histological sections analyzed in a blinded way and how to control for differences in tissue morphology/architecture?

Author's Response: Thank you for raising these important questions. For blinded analysis, the researchers were blinded to group allocation until completion of analysis. Regarding morphology control, we implemented standardized tissue orientation during embedding, consistent sectioning levels verified by anatomical landmarks, and quantitative assessment using ImageJ software with standardized thresholds.

- Line 591, 4 healthy volunteers (2 males...)?

Author's Response: Thank you for pointing this out. The cohort size has been expanded to 10 participants (previously n=4) to enhance statistical power. Detailed demographic data including gender (5 males/5 females) and age range (24-25 years, mean 24 ± 0.5 y) have been added to the revised Methods section (**lines 678**).

- Line 602, IFN instead of INF

Author's Response: Revised (**line 689**).

- Line 610, please remove Nonparametric

Author's Response: Thank you for pointing this out. As suggested, one way ANOVA or Kruskal Wallis test with post hoc testing was applied for multiple comparisons. The statistical method description and all relevant figures and results have been updated accordingly.

- Figure 6c, please remove the word 'concentration' from the y-axis

Author's Response: We thank the reviewer for this suggestion. The figure has been revised.

- Supplementary figure S2 and S10 please remove statistical result for n=2. Statistics can be done from n=3 independent analyses onwards.

Author's Response: Thank you for the valuable comments. We have now performed additional experiments to increase the sample size to 5. All statistical analyses are re-evaluated using these new data, and all figures have been updated accordingly in the revised version (**Fig. S2** and **S10**).

- Supplementary figure S3 and in general: please remove the word 'irrelevant' before allergen OVA, maybe adapt it into model allergen OVA. It may be that the responses found are OVA related and not apply for all allergens. This cannot be told from these studies.

Author's Response: Thank you for the valuable suggestion. We agree that while our findings demonstrate AGEs' potentiating effects on OVA sensitization, further studies are required to determine if this adjuvant effect extends to other dietary allergens. The word 'irrelevant' has been deleted or replaced by 'model' in the revised version.

- Line 119, BALB/c; Line 190, principal components analysis; Line 260, exist; Line 381, lamina

Author's Response: Thank you for pointing these out. These spelling errors have been revised (**lines 130, 288, 435**). For principal components analysis, we would like to clarify that our analysis is principal coordinates analysis, not principal components analysis. Therefore, the term "Principal Coordinates Analysis" is accurate in this context.

Reviewer #2:

- [1]. This review starts with an epidemiological analysis of a North American Nutritional Survey and makes a case for ultra processed foods as a proxy for high dietary AGEs as being a risk factor for allergy. This adds to the literature. It sets the framework for the paper, but is not that novel in itself. There have been 3 more data-sets of nutritional surveys published since. Why were these not used or incorporated? What is noteworthy is the series of in-vivo experiments that explore well recognised factors in food allergy such as microbiome and epithelial junctions and dAGEs ties this together. The work supports the claims. The scientific tools and methods used are robust

and appropriate. No claims are made outside the evidence. There is enough detail in the paper and supplementary material for replication of studies. This collective data should reach a plenary session in our field at a major US or European Meeting. There are a few minimal grammar errors but generally this is well written.

Author's Response: Thank you for the reviewer's insightful comments. The NHANES database is a well-established resource for studying diet-disease associations, providing population-based data on dietary intake and health conditions. In our study, we aimed to leverage this database to investigate the link between AGEs-rich ultra-processed foods (UPFs) and the incidence of food allergy. While previous studies using NHANES have explored the association between UPFs and other allergic conditions (e.g., asthma and dermatitis), *none have specifically examined food allergy due to the lack of relevant data in most NHANES cycles*. Notably, only the 2007-2010 NHANES cycle includes information on food allergy, *making our study the first to systematically investigate the association between AGE-rich UPFs intake and food allergy*. This focus on an understudied aspect expands existing literature and provides novel insights into the role of dietary AGEs in allergic diseases. We believe this contribution adds novelty and value to the field.

- [2]. Line 39: The Th2-stimulating effect of dAGEs involving RAGE-TLR4 crosstalk was finally validated in human peripheral immune cells. I would delete the word finally. You could add "Furthermore" ... at the start of the sentence.

Author's Response: Thank you for the comments. The sentence has been improved (**line 38**).

- [3]. Line 52: It would be strengthened by discussing that emigrants to western countries acquire the risk of food allergy and inhalant allergies when they move in the first 7 years of life or are born there. There are references particularly for the first 7 years for asthma-migrants to Canada and studies in Australia have shown higher rates of FA in first generation immigrants from Asia.

Author's Response: Thank you for the constructive suggestion. We have improved the introduction by discussing the results of migration studies to support the critical role of environmental exposure during critical windows in allergy development (**line 52-55**).

- [4]. Line 61: Dietary AGEs (dAGEs) are a group of heterogeneous compounds formed between reducing sugars and the amino groups of proteins/peptides/free amino acids in food matrices via the non-enzymatic reaction. There are multiple non-enzymatic reactions and pathways to create dAGES.

Author's Response: Thank you for the insightful comments. The word "non-enzymatic reaction" here primarily means the Maillard reaction. We fully agree that dAGEs can be formed through other non-enzymatic reactions and pathways like lipid peroxidation. The text has been improved.

#Sentences added or revised:

(**Lines 64-66**) Dietary AGEs (dAGEs) are a group of heterogeneous compounds formed primarily via the Maillard reaction between reducing sugars and amino groups in proteins **or** peptides within food matrices, as well as through lipid peroxidation and other oxidative pathways.

- [5] Line 159: "Additionally, a higher frequency of TLR4+ and RAGE+ cells in CD45+ cells was also noted in HAGE-groups, indicative of heightened pro161 inflammatory responses in lymphocytes under a high-AGE diet". Do you mean there was higher TLR4 and RAGE expression on CD45+ cells?

Author's Response: Thank you for the comments. Yes, our results demonstrate an elevated TLR4 and RAGE expression in CD45⁺ cells in the HAGE group.

- [6] Line 170: Increased diamine oxidase (DAO). DAO degrades histamine, and this may be induced to cope with either increased histamine from allergies responses or histamine producing microbiota.

Author's Response: We thank the reviewer for this insightful comment. We agree that the rise in DAO may represent a compensatory response to both barrier damage and increased histamine load, highlighting the multifaceted role of AGEs in promoting allergic responses through intestinal and immune mechanisms. This point has been discussed in the revised manuscript.

#Sentences added or revised:

(**Lines 188-190**) In particular, the elevated DAO, also known as histaminase, likely reflects both AGEs-induced enterocyte injury and a compensatory response to heightened histamine levels during allergen sensitization.

- [7] Line 260: can still be observed in RAGE 259 -/- mice. Change "can" to "were" . The sentence is in the past tense. English is a difficult language at times

Author's Response: Thank you for the comments. The sentence has been improved.

- [8] E coli are slow growing species and make AGEs to poison bifidobacteria and lactobacilli which are faster growing. Their enzyme glyoxalase is mentioned in the manuscript, but this is pH dependent and is dramatically reduced if pH is increased (by antacids). Antacids are often used to create animal models of food allergy. dAGES increase the longer food is stored. They form between sugars and proteins or lipids. AGEs will also form with higher blood sugar levels. These will be endogenous formed AGES with human lipids and proteins as all as AGEs made by microbiotia. We should think of a total AGE pool. The western diet is high in sugar and all of this We discussed this in:

Smith PK, Venter C, O'Mahony L, Canani RB, Lesslar OJL. Do advanced glycation end products contribute to food allergy? *Front Allergy*. 2023 Apr 4;4:1148181. doi: 10.3389/falgy.2023.1148181. PMID: 37081999; PMCID: PMC10111965.

We discussed the convergence of RAGE and TLR4 in the above paper - I am not seeking self reference, but this convergence of pathways has been described. It is pleasing to see it shown in animal models of food allergy. It is also important to recognize that Vitamin D -a strong epidemiological and in-vitro factor in food allergy has several intersections with RAGE and TLR4. Again, reviewed in the above paper.

Author's Response: We appreciate the reviewer's insightful comments. In response:

- ① Regarding the formation and sources of AGEs, we appreciate the reviewer's emphasis on the importance of both endogenous and exogenous AGEs in the total AGE pool. While our current study is specifically designed to investigate the role of dietary AGEs in driving Th2-skewed immune responses, we agree that endogenous AGEs and their potential interactions with microbiota represent an important area for future research.
- ② We greatly appreciate the reviewer's suggestion to further discuss the convergence of RAGE and TLR4 pathways. We have expanded our discussion on this point and cited the recommended reference (Smith et al., 2023) to highlight the significance of this interaction in food allergy pathogenesis (*lines 291-294, 425-431*).
- ③ We thank the reviewer for pointing out the potential role of Vitamin D in modulating RAGE/TLR4 signaling. While this is an interesting and important area of research, it extends beyond the scope of our current study. We hope to explore this aspect in future work.

[9] As a closing comment or even at the start of discussion, it is worth mentioning that AGEs appear to have an important role in multiple health disease and probably represent one of the most modifiable factors we have at our disposal. I used AI to help me summarize a series of these (and I checked validity of each reference) .

Advanced Glycation End Products (AGEs) are proteins or lipids that become glycated after exposure to sugars. They accumulate in various tissues and have been implicated in the pathogenesis of numerous diseases, especially those related to aging, diabetes, and oxidative stress. Below is a list of diseases associated with high levels of AGEs, along with corresponding PubMed references.

- *Diabetes Mellitus and Diabetic Complications:*
High blood glucose levels in diabetes lead to increased formation of AGEs, contributing to various complications.

• Reference: o Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes*. 2005 Jun;54(6):1615-25. doi: 10.2337/diabetes.54.6.1615. PMID: 15919781.

- *Cardiovascular Disease*

AGEs contribute to endothelial dysfunction and atherosclerosis, increasing the risk of cardiovascular events.

• Reference: o Ott C, Jacobs K, Haucke E, et al. Role of advanced glycation end products in cellular signaling. *Redox Biol*. 2014 Jan;2:411-29. doi: 10.1016/j.redox.2013.12.016. PMID: 24624331; PMCID: PMC3939615.

- *Chronic Kidney Disease*

Impaired renal function leads to decreased clearance of AGEs, promoting further kidney damage.

• Reference: Forbes JM, Cooper ME. Mechanisms of diabetic complications. *Physiol Rev*. 2013 Jan;93(1):137-88. doi: 10.1152/physrev.00045.2011. PMID: 23303908.

- *Alzheimer's Disease and Neurodegenerative Disorders*

AGEs accumulate in neural tissue, contributing to oxidative stress and neurodegeneration.

• Reference: Li J, Liu D, Sun L, Lu Y, Zhang Z. Advanced glycation end products and neurodegenerative diseases: mechanisms and perspective. *J Neurol Sci*. 2012 Jun 15;317(1-2):1-5. doi: 10.1016/j.jns.2012.02.018. Epub 2012 Mar 11. PMID: 22410257.

- *Osteoporosis*

AGEs affect bone quality by altering collagen properties, leading to decreased bone strength.

• Reference: Ge W, Jie J, Yao J, Li W, Cheng Y, Lu W. Advanced glycation end products promote osteoporosis by inducing ferroptosis in osteoblasts. *Mol Med Rep*. 2022 Apr;25(4):140. doi: 10.3892/mmr.2022.12656. Epub 2022 Feb 25. PMID: 35211757; PMCID: PMC8908347.

- *Rheumatoid Arthritis*

AGEs promote inflammation and joint destruction in rheumatoid arthritis patients.

• Reference: Delrue C, Speeckaert R, Delanghe JR, Speeckaert MM. The Potential Influence of Advanced Glycation End Products and (s)RAGE in Rheumatic Diseases. *Int J Mol Sci*. 2023 Feb 2;24(3):2894. doi: 10.3390/ijms24032894. PMID: 36769213; PMCID: PMC9918052.

- *Skin Aging and Disorders*

Accumulation of AGEs in the skin leads to reduced elasticity and other signs of aging.

• Reference: Paeon H. Reaction of glycation and human skin: the effects on the skin and its components, reconstructed skin as a model. *Pathol Biol (Paris)*. 2010 Jun;58(3):226-31. doi: 10.1016/j.patbio.2009.09.009. Epub 2009 Nov 5. PMID: 19896301.

- *Non-Alcoholic Fatty Liver Disease (NAFLD)*

AGEs may exacerbate liver inflammation and fibrosis in NAFLD.

• Reference: Gaens KH, Niessen PM, Rensen SS, Buurman WA, Greve JW, Driessen A, Wolfs MG, Hofker MH, Bloemen JG, Dejong CH, Stehouwer CD, Schalkwijk CG. Endogenous formation of N^ε-(carboxymethyl)lysine is increased in fatty livers and induces inflammatory markers in an in vitro

model of hepatic steatosis. J Hepatol. 2012 Mar;56(3):647-55. doi: 10.1016/j.jhep.2011.07.028. Epub 2011 Sep 10. PMID: 21907687.

- *Polycystic Ovary Syndrome (PCOS)*

Increased AGEs may influence insulin resistance and hormonal imbalances in PCOS.

- Reference: Diamanti-Kandarakis E, Piperi C, Kalofoutis A, Creatsas G. Increased levels of serum advanced glycation end-products in women with polycystic ovary syndrome. Clin Endocrinol (Oxf). 2005 Jan;62(1):37-43. doi: 10.1111/j.1365-2265.2004.02170.x. PMID: 15638868.

- *Cataracts*

AGEs in lens proteins contribute to lens opacity and cataract formation.

- Reference: Franke S, Dawczynski J, Strobel J, Niwa T, Stahl P, Stein G. Increased levels of advanced glycation end products in human cataractous lenses. J Cataract Refract Surg. 2003 May;29(5):998-1004. doi: 10.1016/s0886-3350(02)01841-2. PMID: 12781289.

- *Erectile Dysfunction*

AGE-induced vascular damage can lead to erectile dysfunction.

- Reference: Celtek S, Cameron N, Cotter M, et al. Pathophysiology of diabetic erectile dysfunction: potential contribution of vasa nervorum and advanced glycation endproducts. Int J Impot Res 25, 1 – 6 (2013). <https://doi.org/10.1038/ijir.2012.30>

- *Inflammatory Bowel Disease (IBD)*

AGEs may play a role in the inflammatory processes of IBD.- CD but not UC.

- Reference: Jiang F, Yang W, Cao Y, Cao X, Zhang Y, Yao L, Cao Q. The contribution of dietary advanced glycation end-products and genetic risk in the development of inflammatory bowel disease: a prospective cohort study. Aliment Pharmacol Ther. 2024 Oct;60(8):1075-1086. doi: 10.1111/apt.18218. Epub 2024 Aug 23. PMID: 39177057.

- *Cancer*

AGEs can may increase risk of cancer and promote tumor growth and metastasis through various mechanisms.

- Reference: Turner DP. Advanced glycation end-products: a biological consequence of lifestyle contributing to cancer disparity. Cancer Res. 2015 May 15;75(10):1925-9. doi: 10.1158/0008-5472.CAN-15-0169. Epub 2015 Apr 28. PMID: 25920350; PMCID: PMC4433613.

Muthyalaiyah YS, Jonnalagadda B, John CM, Arockiasamy S. Impact of Advanced Glycation End products (AGEs) and its receptor (RAGE) on cancer metabolic signaling pathways and its progression. Glycoconj J. 2021 Dec;38(6):717-734. doi: 10.1007/s10719-021-10031-x. Epub 2022 Jan 22. PMID: 35064413.

- *Peripheral Artery Disease*

AGEs contribute to vascular stiffness and impaired blood flow in peripheral arteries.

- Reference:

Garg PK, Biggs ML, Barzilay J, Djousse L, Hirsch C, Ix JH, Kizer JR, Tracy RP, Newman AB, Siscovick DS, Mukamal KJ. Advanced glycation end product carboxymethyl-lysine and risk of incident peripheral artery disease in older adults: The Cardiovascular Health Study. *Diab Vasc Dis Res*. 2019 Sep;16(5):483-485. doi: 10.1177/1479164119847481. Epub 2019 May 8. PMID: 31064218; PMCID: PMC6697602.

- *Chronic Obstructive Pulmonary Disease (COPD)*

AGEs may exacerbate inflammation and tissue damage in COPD.

- Reference: Hoonhorst SJ, Lo Tam Loi AT, Pouwels SD, Faiz A, Telenga ED, van den Berge M, Koenderman L, Lammers JW, Boezen HM, van Oosterhout AJ, Lodewijk ME, Timens W, Postma DS, Ten Hacken NH. Advanced glycation endproducts and their receptor in different body compartments in COPD. *Respir Res*. 2016 Apr 26;17:46. doi: 10.1186/s12931-016-0363-2. PMID: 27117828; PMCID: PMC4847335.

Author's Response: Thank you for this constructive suggestion. We fully agree with the reviewer that highlighting the broader implications of AGEs in health and disease would significantly strengthen the discussion. Accordingly, we have added a closing statement emphasizing the pivotal role of AGEs as a modifiable factor across various health conditions, enhancing the translational relevance of our findings.

Sentences added or revised:

(**Lines 457-460**) Given the involvement of AGEs in multiple diseases, targeting AGEs through dietary or therapeutic interventions may offer a promising strategy for improving public health.

Reviewer #3:

In their manuscript entitled: "Diet rich in advanced glycation end-products misdirected intestinal mucosa immunity to promote food allergy through innate signaling" Zhang et al. establish a link between advanced glycation end-product (AGE) from ultra-processed food (UPF) and the onset and development of allergic diseases. First, using an epidemiologic investigation based on a survey about diet and allergy, they establish a correlation between AGE containing UPS consumption and allergic risk. Then, using different mouse models of food allergy, author demonstrate a link between chronic AGE consumption and inflammation highlighting possible molecular pathways involved. Authors raise an important question to unraveled the important raise of allergic diseases the last decade linking recent hypothesis about barrier degradation and allergy development. Authors show an important amount of data to support their conclusion, however authors should more moderate to not risk to overstate their conclusion. Therefore, some modification may help to improve manuscript impact.

- [1] In the first part of their manuscript, authors used a questionnaire involving a large amount of person to correlate AGE consumption and allergic diseases. In the discussion section, authors should also consider the impact of diet from people with less UPF consumption as a protective effect from allergic disease development. In fact, it has been demonstrated in human and in animal model that fiber or anti-oxidant consumption are protective factor and food allergy or asthma exacerbation development and severity (Moro et al 2006; Ziegler et al., 2008; Braithwaite et al 2014 ; Protudjer et al., 2012). Within the same line, as allergic diseases are based on self-report, authors should be more careful in their conclusion (for instance : L104: “asthma” should be replaced by self-report asthma or asthma symptoms feeling).

Author's Response: We thank the reviewer for this valuable comments. As suggested, we incorporated dietary antioxidant and fiber intake as covariates in our models. After adjustment, the association between high AGE-rich UPFs consumption and allergic diseases remained significant, indicating this relationship is independent of fiber or antioxidant intake (**Fig. 1, Table S1, and Fig. S1**). Additionally, we have revised the manuscript to clarify that allergic disease outcomes were based on self-reported data, replacing terms like “asthma” with “self-reported asthma” or “asthma symptoms” where appropriate. These changes have been reflected in the Methods (**lines 469-491**) and Results sections (**lines 111-124**). We believe these revisions address the reviewer's concerns and strengthen the manuscript.

- [2] Then, in a second part of the manuscript, authors use orally and percutaneous model of food allergy to demonstrate that AGE exposure increase allergy severity and development. Here again, conclusion might be overstated. In fact, authors clearly demonstrate an inflammatory effect of AGE exposure regarding IgG1, Total IgE, mast cell in colon, CD8 infiltrate, Th2 cells and Treg cells. However, increase of allergic diseases severity is less clear as allergy score (based on subjective parameters such as scratching or behavior) is not increase comparing SD FA to HAGE FA. Similarly, specific IgE, Treg and CD4 T cells remains similar between SD and HAGE groups in allergic conditions. Other allergic marker based on allergic symptoms manifestation measurement such as histamine, mMCP1, gut permeability may help to reinforce effect of AGE in allergic conditions. Nevertheless, modification has to be made in results description such as L138, L144 and L163 where HAGE mice seems to be in a low inflammatory condition more than in allergic exacerbation both in oral and percutaneous model.

Author's Response: We sincerely thank the reviewer for their constructive feedback. We agree that high-AGE diets may not significantly increase susceptibility to allergy initiation. However, our data robustly demonstrate that high-AGE diets exacerbate allergic inflammation, as evidenced by increased mast cell infiltration and degranulation in the intestine (elevated MCT and MCC

expression in HAGE-OVA vs. SD-OVA), elevated Th2 cell proportions and NF- κ B activation in CD4⁺ T cells in MLNs. Even in non-allergic mice, HAGEs induced systemic inflammation, as shown by elevated total IgE levels, increased mast cell accumulation, and Th2/Treg imbalance. We have revised the text to avoid overstating conclusions about "allergy severity" and instead focus on the exacerbation of allergic inflammatory responses (*lines 140-152, 175-177*). Again, we appreciate the reviewer's insightful comments that have helped us refine our manuscript.

- [3] In a third part of their manuscript, authors demonstrate the adjuvant effect of AGE consumption. Using mouse model of adjuvant free orally sensitized mice, authors demonstrate an increase of global gut permeability after 12 weeks of AGE diet. However, they showed DAO and Lactic acid (Figure 3d and e) only in HAGE-OVA fed mice, also it is difficult to measure impact of OVA bound to AGE without depicting AGE fed mice on the same graph (as for TJ and TLR expression). Similarly, for microbial diversity measurement (Figure 3i and j), mice fed with AGE, SD, SD-OVA and HAGE-OVA at week 16 should be depicted. Then, using BSA-AGE as an adjuvant in orally sensitized food allergy to OVA model, authors demonstrate FA development in AGE-OVA sensitized mice but not in OVA sensitized mice based on a slight increase in body temperature drop, sIgE level and allergy score. However, as AGE adjuvant effect seems restricted to orally sensitization, it would be of interest to compare adjuvant effect of AGE with common adjuvant known for oral sensitization such as cholera toxin deciphering the potential adjuvant effect of AGE to a global inflammatory effect as suggested by the increase of Th1 cells (Figure 4g).

Author's Response: We thank the reviewer for their insightful comments and suggestions. Below, we address each point in detail:

① *Barrier-damaging markers:*

We have now supplemented the results with control group data for TJ-proteins and RAGE-/TLRs expression in the intestine for both long-term intervention models (*new data in Figure 3f-g, and Supplementary Fig. S7*). Results confirmed that the control group (mice fed a standard diet without OVA) showed significantly higher levels of TJ-proteins compared to the HAGE group. This supports that the observed barrier dysfunction is primarily driven by the HAGE diet rather than OVA alone. In parallel, we observed that the expression levels of RAGE and TLR4, but not TLR2 were significantly up-regulated in HAGE-fed mice relative to the SD controls. This suggests that the HAGE diet may specifically activate the RAGE/TLR4 signaling pathway, which could contribute to the observed barrier dysfunction and inflammatory response.

However, due to the depletion of serum samples, we were unable to include control samples for the DAO and D-lactic acid assays. While we believe that the current results provide sufficient

evidence to support our conclusions. Future studies will prioritize the inclusion of these controls to further validate our findings.

② Microbial diversity analysis:

For microbial analysis, our hypothesis, which was ultimately supported by the data, was that a 12-week HAGE diet induced microbial dysbiosis and predisposes mice to subsequent allergen sensitization. This is supported by the strong associations between the gut microbiota composition at week 12 and subsequent barrier damage and allergic inflammatory markers at 16th week. While we analyzed microbial diversity at week 16 and found no significant differences between groups (see Fig. r1 below), these results further indicated that the early changes in microbiota induced by HAGE play a critical role in driving later allergic responses.

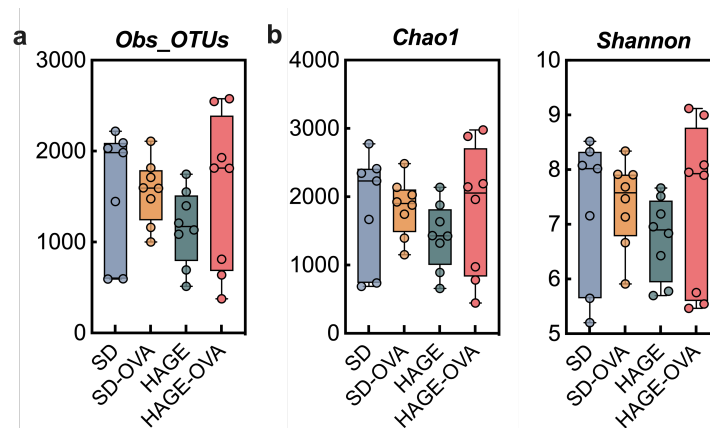


Figure r1. Intestinal microbiota diversity of mice at the 16th week. **a**, Total OTUs. **b**, α -diversity indices.

* Mice received either a control standard diet (SD) or a heat-treated high-AGE diet (HAGE) for 12 weeks before induction of experimental food allergy. Feces at 16 weeks were collected for analysis.

Results are expressed as mean $\pm$ SEM ($n = 7-8$).

② AGE's adjuvant effect:

The BSA-AGE model is a novel, adjuvant-free sensitization model developed in our study. It demonstrates that AGEs alone can enhance sensitization to by-stander allergens, as evidenced by significant changes in allergic symptoms and allergen-specific immune responses. Importantly, this process is oral-mucosal dependent, further supporting our conclusion that AGEs promote allergic sensitization through intestinal barrier disruption and inflammation. While the reviewer's suggestion to compare AGEs with other adjuvants (e.g., CT) is intriguing, this was beyond the scope of the current study. However, we agree that exploring AGEs' global inflammatory effects is an important direction for future research, and we are actively pursuing this in ongoing studies.

- [4] Finally, authors explore molecular pathways involves in AGE adjuvant effect using TLR4 and RAGE KO mice and show that TLR4 KO as well as RAGE KO mice do not develop allergic

reaction as suggest by the allergy score, body temperature drop and specific IgE (Figure 5a-e). As no allergic disease is observed in AGE as well as AGE-OVA experimental condition neither in TLR4 or RAGE KO mice, it is unclear whether TLR4 and/or RAGE is specifically involved in the allergic process. It would have been interested to include OVA sensitized and challenged mice in TLR4 and RAGE KO mice to reinforce the role of AGE not only in inflammation but in the allergic process. Authors should discuss the high level of specific IgE observed in RAGE KO mice (Figure 5d). Similarly, PBMC should be exposed to an allergen induce changes in bacterial metabolite such as OVA to measure the impact of AGE exposure impact on bacterial metabolites in the allergic context.

Author's Response: We thank the reviewer for their insightful comments. Below, we address these concerns point by point:

① *Specificity of TLR4 and RAGE in the allergic process:*

We appreciate the reviewer's suggestion to include OVA-sensitized and challenged mice in TLR4 and RAGE KO models. While this would provide additional insights, our study focuses on the adjuvant effect of dietary AGEs (dAGEs) in promoting allergic inflammation, rather than OVA alone. Our data show that BSA-AGE significantly amplifies OVA-specific Th2 responses and allergic inflammation in wild-type mice, and this effect is abolished in TLR4 and RAGE KO mice, underscoring the critical role of these receptors in dAGEs-mediated sensitization. To address the reviewer's concern, we have revised the discussion to further emphasize the specific contribution of TLR4 and RAGE to AGEs-induced allergic inflammation.

#Sentences added or revised:

(**Lines 425-431**) Interestingly, in RAGE-deficient mice, we observed up-regulation of TLR4 and its adaptor molecule MyD88, along with elevated IL-33 levels and a trending higher GATA3 expression in the intestine. However, activation of TLR4 alone is insufficient to drive the full Th2 immune response required for allergic sensitization. These findings suggest that TLR4 and RAGE signaling may contribute to distinct stages of dAGEs-induced allergic inflammation: TLR4 drives early barrier disruption and localized inflammation, while RAGE is essential for later-stage immune cell activation and allergen-specific responses.

② *High levels of specific IgE in RAGE KO mice:*

We thank the reviewer for noting the specific IgE levels in RAGE^{-/-} mice. While RAGE deficiency reduced overall allergic responses, IgE levels were comparable between AGE and AGE-OVA groups in RAGE^{-/-} mice, unlike in wild-type mice where AGE-OVA induced higher OVA specific IgE responses. This suggests that RAGE is essential for amplifying OVA-specific IgE production in the presence of AGEs, while alternative pathways (e.g., TLR4 signaling or other inflammatory

mechanisms) may contribute to the baseline IgE production in the absence of RAGE. We have expanded the discussion to clarify this point.

#Sentences added or revised:

(**Lines 271-274**) Interestingly, while RAGE^{-/-} mice were protected from AGEs-induced allergic symptoms, they showed higher baseline IgE levels compared to WT and TLR4^{-/-} mice. This may result from compensatory mechanisms, such as enhanced TLR4 signaling, which sustain low-level IgE production but fail to drive OVA-specific IgE enhancement or full allergic inflammation.

③ *Group design in PBMC experiments:*

In the PBMC experiments, we aimed to elucidate the direct effects of AGEs and associated metabolites on immune responses, particularly in type 2 inflammation. To better address this, we have now included FM-SD (a fecal supernatant from control-fed mice) as a control. The results show that FM-HAGE, but not FM-SD, significantly enhanced PBMC activation and cytokine production, confirming the effects are attributable to AGE-related bacterial metabolites (**Fig. 6, Fig. S14, lines 306-318**). Regarding the suggestion to add metabolites from OVA-sensitized mice, we believe this would introduce additional variables (e.g., allergen-specific immune activation and microbiota interactions) that could complicate the interpretation of our results. Our current experimental design, we believe, provides clear evidence that AGEs drive gut microbiota dysbiosis, potentially eliciting type 2 inflammation and shaping a pro-inflammatory microenvironment predisposing to allergic sensitization. We sincerely thank the reviewer for these insightful comments, which have helped strengthen our study.

Specific minor comment

- [1] L120: Authors should add the starting point of the 12 weeks AGE exposure as Figure 2a show a negative starting point meaning pregnant mice were exposed? Similarly, age of mice should mention in the material and methods section.

Author's Response: Thank you for pointing this out. We have clarified the timeline of the 12-week AGE exposure in the revised **Fig. 2a, 3a**, and **S5**. The age of mice (4~6 weeks) has been stated in the Method section (**line 494**). We hope these revisions address your concerns and improve the clarity of our study design.

- [2] Figure 4b should be cited before figure 4c. Text might modify or panel order in Figure4.

Author's Response: Thank you for pointing this out. We have adjusted the panel order in Figure 4 to align with the sequential citation of subfigures in the text.

Detailed Response to the Reviewers

Reviewer #1:

In the rebuttal to my comments and the revised manuscript, authors carefully addressed all issues raised. In particular the addition of the OVA specific T-cell analyses using tetramers is interesting and convincing that indeed a systemic ova specific Th2-cell response is generated in the AGE + OVA sensitization model.

[1]. Also intestinal mast cell protease expression was assessed. With regard to this staining, it is a bit hard to appreciate if indeed this shows enhanced mast cell activation and degranulation (quality is not very sharp). As the immunohistochemistry pictures do not show (degranulating) mast cells. The expression levels of MCT and (possibly) MCC (but MCC was also already high in the controls which would not be expected) do seem to be affected. It is unclear at which enlargement these pictures were taken. Hence these additions are interesting but care should be taken in making to strong conclusions.

Author's Response: We sincerely appreciate the reviewer's insightful comments regarding mast cell analysis. As mast cell protease (MCT and MCC) expression are well-established biomarkers of mast cell activation state that are widely recognized in the field, we have addressed the concerns as follows:

Image Clarity Enhancement

- ⑩ Added scale bars (now indicated in revised Figure S3c)
- ⑩ Clearly marked mast cells with black arrows (consistent with published methodologies [1,2])
- ⑩ Included higher-magnification insets to demonstrate degranulation morphology.

Biological Context

The elevated MCC expression in HAGE-fed mice compared to SD controls can be explained by the fact that long-term AGE exposure primes the immune system and enhances its responsiveness to subsequently encountered allergens, in consistence with the observed high total IgE and mast cell accumulation in HAGE groups.

We hope the revised figures and explanations now provide a more comprehensive understanding of our observations.

[2]. Fig 4 y-axis states total IgEn should this be total IgE?

Author's Response: We apologize for this mistake. The figure y-axis legend has been revised.

[3]. Fig S3 y-as should probably state colon instead of jejunum.

Author's Response: We apologize for this mistake. The figure y-axis legend has been revised.

References

- [1] Rozsivalova, K., Pierzynova, A., Kratochvilova, H., Lindner, J., Lipš, M., Kotulák, T., ... & Kučera, T. (2020). Increased number of mast cells in Epicardial adipose tissue of cardiac surgery patients with coronary artery disease. *Physiological Research*, 69(4), 621.
- [2] Pavlova, T. V., Pilkevich, N. B., Bessmertnyi, D. V., Pavlov, I. A., Atiakshin, D. V., & Pavlova, L. A. (2021). Evaluation of new immunohistochemical approaches for the study of kidney tumors in geriatric. *Archives of Razi Institute*, 76(4), 1107.