Gut microbiota modify risk for dietary glycemia-induced age-related macular degeneration

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**ABSTRACT**
Age-related macular degeneration (AMD) is a leading cause of blindness world-wide. Although the etiology of AMD is multifactorial, diet and nutrition have strong epidemiologic associations with disease onset and progression. Recent studies indicate a role for gut microbiota in development of AMD in mouse models and in some forms of human AMD. We previously found that consuming lower glycemia diets is associated with protection against AMD in humans and switching from higher to lower glycemia diets arrests AMD phenotypes in mice. Gut microbiota populations and circulating microbial cometabolites were altered in response to dietary carbohydrates, indicating a gut-retina axis. Here we explore additional gut microbiota-AMD interactions that point toward pathogenic roles for some gut microbiota families, including Ruminococcaceae and Lachnospiraceae, and individual members of Turicibacteraceae, Clostridiaceae, and Mogibacteriaceae. We also speculate on potential mechanisms by which gut microbiota influence AMD, with the objective of devising new AMD diagnoses and treatments.

**KEYWORDS**
Age-related macular degeneration; glycemic index; gut microbiome; microbial cometabolite; biomarker

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**INTRODUCTION**
There are two forms of AMD: dry and wet. Both lead to visual impairment and potentially blindness due to death of photoreceptors. The latter, also called neovascular AMD, occurs when the choroidal blood supply in back of the eye invades into the photoreceptor layer. The vast majority of AMD is of the dry type. Our understanding of AMD has been greatly enhanced by epidemiologic studies that indicated important roles for environment, genetics, and diet in AMD morbidity. Strikingly, many of the risk factors for AMD like adiposity, smoking, and consumption of Western-style high glycemic index dietary patterns are also strong risk factors for CVD and diabetes, albeit they involve different tissues and often present at different times of life. The importance of dietary glycemic index has been validated in wildtype C57BL/6j mouse models of AMD wherein mice fed high glycemic index (HG) diets develop hallmarks of AMD, while mice fed low glycemic index (LG) diets do not. HG and LG diets used resemble standard rodent chow and are isocaloric, varying only in the type of starches used. The HG dietary starch consists of 100% amylpectin, a rapidly digested starch, while the LG dietary starch consists of 70% amyllose/30% amylpectin, which is slowly digested. The effective glycemic index difference between these diets is approximately 55%, mirroring differences between the HG and LG breakfast cereals Cornflakes® and All-bran®. We recently demonstrated that switching diets from HG to LG diets midway through aging was able to prevent development of AMD and reverse accumulation of damage in the retina. Mice fed LG diets efficiently maintained their weights and were normoglycemic, whereas mice fed HG diets gained weight and become hyperglycemic and glucose intolerant.

We evaluated gut microbiota in LG and HG mice by 16S rDNA pyrosequencing and discovered alterations to gut microbiota populations that were mediated by diet and reversible by dietary change. We also evaluated the plasma and urine metabolomes and identified a large number of ‘microbial cometabolites’,
metabolites whose levels change in response to micro-
bacteria, that were enriched in LG mice and that associ-
ated with protection against AMD features. We hypothesized that such metabolites, which included sero-
totonin, hippurate, 4-hydroxyphenylacetate, trypto-
phan, trimethylamine, tyrosine, and indoxylsulfate,
may alone, or in combination, mediate retinal neuro-
protection, involving the neuroretina and the support-
ing retinal pigmented epithelial cells. By analogy with 
the gut-brain axis, we referred this signaling pathway 
as the gut-retina axis, with the recognition that it may 
not be distinct from the gut-brain axis, as the retina is 
a part of the central nervous system.

The gut microbiome in AMD

Using statistical approaches, we evaluated micro-
bacteria at multiple taxonomic levels to determine
whether they showed associations with AMD, with 
diets, or with dietary change from HG to LG.7 As 
expected, some microbiota showed strong associa-
tions with diet. Enrichment with Bacteroidetes was 
associated with LG diet, in particular the S24–7 
family and the Bacteroidia class, whereas high lev-
els of Firmicutes were associated with HG diet, 
particularly the Clostridia class. We also showed 
that many groups of microbiota were associated 
with AMD, some of them in a diet-independent 
fashion. The associations were both positive and 
negative. Elevated populations with Clostridia and 
Bacilli classes were associated with greater risk for 
AMD. More Bacteroidia and Erysipelotrichi were 
associated with protection against AMD.

While these results were being prepared for pub-
lication, two additional studies were published that 
further enhanced our understanding about the con-
nection between gut microbiota and AMD. The 
first was a mouse laser-induced model of neovas-
cular AMD (wet AMD), in association with a high fat 
diet.10 The authors determined that high fat diets 
exacerbated AMD in a gut microbiota-dependent 
fashion. Furthermore, mice fed high fat diets 
showed increased gut permeability and systemic 
inflammation. Increased inflammation exacerbates 
choroidal neovascularization in the eye, which is 
what the authors found with high fat diets. The 
increased gut permeability associated with high fat 
diet was not mitigated via antibiotic treatment, but 
fecal transplantation from mice fed normal diets to 
mice fed the high fat diets, in conjunction with 
altering the gut microbiota, attenuated the neovas-
cular response to laser injury. The major groups of 
microbiota that responded to diet and fecal trans-
plantation were members of Bacteroidetes, which 
were higher in normal diets and members of Firmi-
cutes and Proteobacteria, which were higher in 
high fat diets, consistent with previous literature 
and with the trends seen in our HG and LG mice.

Although mouse models of AMD have greatly 
enhanced our understanding of mechanisms of disease 
pathogenesis, including the role of gut microbiota, 
each model has limitations.11 In the case of our work 
and the study described above, diets were critical com-
ponents and drivers of disease. Since the diet exerts 
such a strong and only partially reversible effect on 
gut microbiota, it may be impossible to definitively 
tease apart the influence of gut microbiota on AMD 
from other dietary effects. Furthermore, mouse micro-
bacteria and diets are quite dissimilar from those in 
humans. Therefore it is critical to both explore new 
mouse models of AMD, preferably in gnotobiotic 
mice, where the microbiome can be fully controlled 
and humanized. It is also essential to explore the gut 
microbiome in human AMD, which is just now 
emerging.12

In a first description of the gut microbiome in 
human AMD, 12 individuals with neovascular 
AMD were compared to 11 control individuals for 
gut microbiota composition.12 Even with this small 
sample size, principal component analysis demon-
strated differences in microbiota composition 
between cases and controls. Further taxonomic 
associations identified bacteria enriched in cases or 
enriched in controls. Control individuals had 
higher amounts of Bacteroides, with the species 
Bacteroides eggerthii being significantly associated 
with controls. This finding is consistent with our 
finding of Bacteroidia being associated with LG 
diet and protection against AMD.7 AMD individu-
als had higher amounts of Anaerotruncus and 
Oscillibacter and at the species level Ruminococcus 
torques and Eubacterium ventriosum.12 All of these 
species or genera belong to the order of Clostri-
diales, which we observed to be associated with 
HG diet and AMD in our animal studies.7

At this time, it is premature to make generalizations 
about microbiome differences in AMD patients versus 
control individuals. The above study was quite small
and the subjects were recruited from a single hospital in Switzerland. Future cohorts that encompass other forms of AMD will likely yield different results with different sets of limitations. Only after extensive phylogenetic and metagenomics studies are reported will we have a more definitive sense of how changes to the gut microbiota influence risk, progression, and phenotypes of AMD in people.

**Associations of HG-induced AMD with individual taxonomic units**

In our study of AMD related to HG and LG diets, as in many other microbiome studies, we grouped phylogenetically related taxa together and assessed association with AMD. This analytic approach is desirable because we sought to identify a consensus notion for the kinds of bacteria that work through similar functional pathways to influence AMD, while minimizing the number of multiple comparisons for statistical purposes. A shortcoming of this approach is that our analysis missed out on identification of microbiota that may have prominent effects, but that are negated by other highly-related members that have no effect or an opposite effect. As we learn more about the human microbiome, it has become clear that bacteria in closely-related species, or even different strains of the same species, can carry out very different functions within their host.

We performed an ROC analysis using the same binary classifications of affected versus unaffected that we did in the original study at the level of individual metabolites (affected = retina damage score > 3). We included all of the OTUs identified in our sequencing analysis, using a false discovery rate cutoff of 0.1. The full list, grouped by taxonomic identification is shown in Table 1. Our analysis indicated multiple OTUs that could efficiently categorize affected from unaffected mice.

Shown in Figure 1 are examples of the top 3 OTUs associated with AMD and the top 2 OTUs associated with the non-disease state. These examples include 4 different families of microbiota and are representative of the remaining OTUs. Of the 39 OTUs that met statistical cutoffs, all but 3 were associated with AMD, and every categorized OTU belonged to the Firmicutes phylum. Several OTUs distinguished AMD from non-AMD nearly perfectly.

Grouping these OTUs taxonomically revealed an apparent enrichment for two particular families of the Clostridiales order, Ruminococcaceae and Lachnospiraceae. We applied enrichment analysis using the hypergeometric distribution to determine whether these groupings were likely chance events or not. As shown in Table 2, Ruminococcaceae were significantly enriched among AMD-predictive OTUs and Lachnospiraceae just missed statistical significance. The significant enrichment for Clostridiales with AMD was similar to our previous

| OTU | Taxonomy | AUC | P value |
|-----|----------|-----|---------|
| OTU 11 | c_Bacillio_Turicibacterales; f_Turicibacteraceae | .983 | 3.09E-06 |
| OTU 28 | c_Clostridia_o_Clostridiales; f_Clostridiaceae | .972 | 5.02E-06 |
| OTU 126 | c_Clostridia_o_Clostridiales; [Mogibacteriaceae] | .941 | 2.03E-05 |
| OTU 418 | c_Clostridia_o_Clostridiales; f_Ruminococcaceae | .917 | 5.65E-05 |
| OTU 132 | .910 | 7.50E-05 |
| OTU 266 | .863 | 4.54E-04 |
| OTU 189 | .844 | 8.93E-04 |
| OTU 187 | .840 | 1.01E-03 |
| OTU 207 | .840 | 1.01E-03 |
| OTU 324 | .835 | 1.20E-03 |
| OTU 264 | .809 | 2.82E-03 |
| OTU 260 | .802 | 3.51E-03 |
| OTU 18 | .792 | 4.82E-03 |
| OTU 258 | .783 | 6.24E-03 |
| OTU 555 | c_Clostridia_o_Clostridiales | .885 | 1.95E-04 |
| OTU 52 | .868 | 3.75E-04 |
| OTU 203 | .859 | 5.14E-04 |
| OTU 384 | .847 | 7.91E-04 |
| OTU 209 | .842 | 9.48E-04 |
| OTU 65 | .833 | 1.27E-03 |
| OTU 110 | .816 | 2.26E-03 |
| OTU 208 | .816 | 2.26E-03 |
| OTU 82 | .809 | 2.82E-03 |
| OTU 399 | .807 | 2.98E-03 |
| OTU 314 | .792 | 4.82E-03 |
| OTU 86 | c_Clostridia_o_Clostridiales; f_Lachnospiraceae | .851 | 7.01E-04 |
| OTU 128 | .847 | 7.91E-04 |
| OTU 155 | .845 | 8.41E-04 |
| OTU 206 | .844 | 8.93E-04 |
| OTU 235 | .842 | 9.48E-04 |
| OTU 163 | .840 | 1.01E-03 |
| OTU 115 | .819 | 2.02E-03 |
| OTU 137 | .806 | 3.15E-03 |
| OTU 499 | .785 | 5.93E-03 |
| OTU 141 | c_Bacillio_Lactobacillales; f_Enterococcaceae | .839 | 1.07E-03 |
| OTU 345 | Unknown | .830 | 1.43E-03 |
| OTU 15 | c_Actinobacteria; o_Bifidobacteriales; f_Bifidobacteriaceae | .208 | 4.82E-03 |
| OTU 195 | c_Clostridia_o_Clostridiales | .108 | 1.48E-04 |
| OTU 13 | c_Erysipelotrichi; o_Erysipelotrichales; f_Erysipelotrichaceae | .104 | 1.30E-04 |
However, the large number of OTUs that belonged to Ruminococcaceae and Lachnospiraceae was surprising, given that in aggregate neither family was significantly associated with AMD, even though they had some association with HG diets. Considering the large number of Ruminococcaceae and Lachnospiraceae OTUs that did not show significant association with AMD, one possible explanation is that these particular family members have distinct functions not shared by other family members, or might be related to one particular species like *Ruminococcus torques*, which is reported to be associated with human AMD. Alternatively, there could be a competitive interaction for a similar niche between highly related beneficial and pathogenic bacteria.

Other OTUs associated with AMD or the non-AMD state were reflected in the larger taxonomic analysis from our previous analysis. For example, the Bacilli class association with AMD is accounted for by OTU 11 and OTU 141 and the Erysipelotrichales order association with protection from AMD is accounted for by OTU 13. OTU 13 and OTU 11 represented the highest and lowest AUCs from our ROC analysis and neither demonstrated strong dietary associations. Further elucidation of the species and functions represented by these OTUs will yield insights into the nature of their AMD associations.

### Prospects of microbial cometabolites as indicators of AMD status

Gut microbiota carry out a broad array of activities in the body that affect our systematic physiology including effects on inflammation, metabolism, and the

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**Table 2.** Taxonomic enrichment of OTUs that classify AMD from non-AMD mice (AMD-ROC).

| OTUs       | p_Firmicutes | o_Clostridiales | f_Ruminococcaceae | f_Lachnospiraceae |
|------------|--------------|-----------------|-------------------|-------------------|
| AMD-ROC    | 36           | 35              | 33                | 11                | 9 |
| Total      | 592          | 442             | 403               | 84                | 94 |
| $P$ value  | 2.4E-04      | 5.4E-04         | 0.00504           | 0.0552            |
Although we are still unraveling the multiple mechanisms involved, it is clear from metabolomic studies that gut microbiota influence the synthesis and levels of many metabolites, which we collectively refer to as microbial cometabolites. Some cometabolites are only produced by gut bacteria (e.g. short-chain fatty acids). Other cometabolites reflect bacterial-specific modifications of host metabolites (e.g. trimethylamine is produced by gut microbiota from host choline or L-carnitine). Yet other cometabolites are largely synthesized by our host cells in response to bacterial signals (e.g. serotonin in response to bile acids). Bacterial production of cometabolites may utilize metabolic biosynthetic pathways that are distinct from the synthetic pathway that is exploited to make the same metabolite in mammals (e.g. chorismate, a precursor of tryptophan). Systematic measurements of microbial cometabolites reveal a biological readout of gut microbiota function independently from standard microbiome metagenomics analyses that may provide novel insights into host-microbiota functional interactions.

In our studies evaluating HG-induced AMD, we identified seven microbial cometabolite that negatively associated with AMD and were all present at higher abundance in LG-fed mice than HG mice. Six of these metabolites were identified in the urine samples taken several months before evaluation of AMD. Therefore, each metabolite represents a prospective biomarker for non-invasively monitoring AMD development. We are determining if these associations would translate to different mouse AMD models, which might impact gut microbiota in a different fashion, or in human AMD. Each metabolite offers its own potential link to metabolic pathways that may alter risk for AMD incidence or progress, as well as to the gut bacteria and gut bacterial gene products that contribute to its production. It is also possible that a metric based upon combinations of these metabolomic and microbiome data will provide even greater predictive capability.

In our metabolomics study, we did not identify any microbial cometabolites that were present at higher abundance in HG mice or that positively associated with AMD. However the analysis presented above certainly implicates HG-enriched microbiota in having specific pathogenic roles in AMD. We believe these mechanisms are distinct from the neuroprotective function of LG-enriched microbiota or their associated metabolites, although we can’t rule out as yet unidentified AMD-associated microbial cometabolites. Pathogenic mechanisms may include increased inflammation and gut permeability, as suggested by high fat-diet exacerbated neovascular AMD. The mechanisms may be indirect via secondary effects on host metabolism, such as an obesogenic function or altered insulin signaling. Ongoing experiments using specific depletion methods and microbiota transfer are interrogating roles for microbiota, their products, and interactions with host in the etiology and treatment of AMD.

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**Disclosure of potential conflicts of interest**

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