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# Aging, Inflammation, and Gut Microbiota in Mice

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**Aging, Inflammation, and Gut Microbiota in Mice**

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**at Bowling Green State University in partial fulfillment of the**

**requirements for graduation**

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## Abstract

Inflammation and gut microbiota are two areas of study that can be linked to aging in the body. As a person ages, systemic inflammation tends to increase, and changes in microbiota in the gastrointestinal system occur as well.

**Purpose:** The purpose of this study was to determine the effects that aging has on selected inflammatory markers in the body, and how gut microbiota changes with age in diabetic mice.

**Methods:** Type II diabetic (T2D; *dbdb* or B6. *Leprdb/J*; Jackson Labs, Bar Harbor, Maine) mice were studied in this experiment. Mice were 10 weeks old: Y1, Y2, and Y3 ( $n=3$ ); and 10 months old: O1 and O2 ( $n=2$ ). The mice were fed *ad libitum* normal chow diet during the study. Bone-derived macrophages were extracted from the femur and analyzed for polarity. In addition, fecal samples were collected from the mice so that the gut microbiome of the mice could be analyzed for changes in bacterial populations.

**Results:** In the tests performed to evaluate macrophage polarity, tumor necrosis factor alpha (TNF- $\alpha$ ) was found in higher levels in the young than in the older mice. Inducible nitrogen oxide synthase (iNOS) was found to be highest in O2, and of similar levels in O1, Y1, and Y2. Arginase 1 (ARG 1) was also highest in O2, and of similar levels in O1, Y1, and Y2. Finally, the mannose receptor (MR) on macrophages was higher in the O1 and O2 than the Y1 and Y2. The microbiome results showed that Firmicutes were higher in the older mice than the young, and Bacteroidetes were lower in the old mice, and higher in the young mice. Due to a small sample size statistical analysis was not performed in this observational pilot study. A follow-up study will be completed so that further results can be collected.

**Conclusions:** Taken together, the results for tests on inflammation were somewhat inconclusive, as the results for the amount of biomarkers were inconsistent. One of the older mice did not have the expected phenotype, likely leading to variable levels of these biomarkers, which makes it difficult to come to a conclusion about inflammation and aging in this pilot study. However, the results for

microbiome from this experiment are similar to a study (Cui, Zhao, Hu, Zhang, & Hua, 2017) which found that high Firmicutes populations and low Bacteroidetes populations were correlated with CHD.

Keywords: Aging, coronary heart disease (CHD), type II diabetes (T2D), low-grade inflammation, macrophage polarization, tumor necrosis factor-alpha (TNF- $\alpha$ ), gut bacteria, microbiota

## **Introduction**

Biological aging has many impacts on the body and its functions. Aging affects all living beings, impacting the various ways that systems interact within the body. One common effect of aging is an increase in systemic inflammation in the body. Inflammation is a response in the body that occurs due to injury or damage in tissues. In many cases it is helpful for healing as it brings leukocytes to areas in need of attention, so that foreign pathogens in the body can be removed. In healthy individuals, most of the inflammation observed is acute, and ensures that the body stays free of disease. However, in aged individuals, a specific type of inflammation called low-grade inflammation is found in higher levels. Low-grade inflammation occurs when inflammatory mediators are signaled too much and too often. This specific type of inflammation is associated with diseases including dementia, rheumatoid arthritis, and cardiovascular disease, which are more common in the elderly (Wadley, Veldhuijzen van Zanten, & Aldred, 2013).

Higher levels of low-grade inflammation in older individuals can be explained because inflammatory markers tend to increase with age. In a study by Wu, Pae, Guo, Cui, Merrill, & Meydani (2007), older mice were found to have more inflammatory mediators and fewer anti-inflammatory receptors. Similarly, a study by Bottino, Lopes, Oliveira, Mecnas, Clapauch, & Bouskela, (2015), observed healthy elderly women for signs of inflammatory biomarkers compared to young women. It was found that inflammatory biomarkers were increased in elderly women, despite their overall good

health. This seems to suggest that aging has an independent effect on increased inflammation in the body, apart from disease and other complications that may arise later in life. In general, as age increases, so do the number of inflammatory mediators, reflecting the amount of systemic inflammation. This increase in inflammation can also be attributed in part due to muscle loss that occurs with age (known as sarcopenia) as well as lower levels of androgens also associated with aging (Wadley et al., 2013).

TNF- $\alpha$  is a proinflammatory cytokine which serves as a biomarker that is often used to measure the inflammatory status of animals. Higher levels of TNF- $\alpha$  indicate higher levels of inflammation in body tissues. (Keylock, Woods, Wallig, & Dipietro, 2008). TNF is one of the biomarkers that was measured in this study.

Not only is inflammation associated with age, but it is also associated with type II diabetes (T2D) and obesity. Those who are obese have been shown to have higher amounts of CD4+ T cells in their adipose tissue (Chang, Rao, & Zhong, 2017). T helper cells are responsible for producing proinflammatory cytokines in vital organs such as the pancreas. In the study by Chang et al. (2017), these proinflammatory cells were also more likely to be in an activated state in obese individuals. T cells are further important in inflammatory processes, as they induce macrophages to proinflammatory states in adipose tissue, the liver, and pancreas. Macrophages are cells that function in the immune response of an organism by engulfing foreign pathogens, and defending the host. These also play a role in regulation of inflammation, can secrete cytokines, and have the ability to polarize into M1 or M2 cells, based on signals received from the environment. M1 cells occur in proinflammatory state, and M2 cells occur in an anti-inflammatory state (Xiaoyuan, Xiangfeng, & Qiu, 2017). In this study, the bone derived macrophages were removed from the femurs of the mice and allowed to polarize so that the state of inflammation in the mice could be determined and analyzed.

Mice which spontaneously develop T2D at an early age (*dbdb*) are expected to become obese as well. Obese mice are expected to have higher levels of TNF- $\alpha$  in the blood and certain tissues, along with increased insulin resistance. Insulin resistance is a known mechanism that causes T2D, and TNF is a known inflammatory marker, so the association between inflammation and diabetes has been well established (Palmer & Kirkland, 2016).

Gut microbiota is another area of study related to aging. The microbiota found in the gut include bacteria, with some eukaryotes, viruses, and archae. Gut bacteria are important for producing enzymes for digestion, and the bacteria present are changeable over time. Past studies have shown that those who are obese have less diversity in their gut bacteria than those of a healthy fat percentage (Sankar, Raoult, Lagier, Pontarotti, & Fournier, 2015). This seems to suggest that there is a correlation between health and the gut bacteria observed.

Gut bacteria have also been found to be involved in the immune function of humans. In fact, much of the immune system relies on the microbiome of the organism, including inflammatory response to infection and the presence of foreign antigens. There is a mutual beneficial relationship existing between the bacteria and the host in the gut, which helps to aid immune function. Further, a majority of the body's lymphocytes, which play a role in keeping the body from infection, are located in tissues related to the gut (Buford, 2017). Considering the immune system plays a large role in keeping the body healthy, gut bacteria are very important in the overall health of an individual.

Diet and exercise affect gut microbiota. For example, high fiber diets have been shown to yield lower amounts of Bacteroidetes than low fiber diets. Exercise has been shown to reduce certain bacteria that are associated with inflammation and obesity including *Erysipelotrichaceae* and *Turicibacteraceae* families of bacteria (Nehra et al., 2016). Therefore, it is important to understand the effects of gut microbiota on the body, because it is possible that with proper exercise and diet, the bacteria

populations that keep individuals healthy could be increased, and the bacteria that increase disease and inflammation could be decreased.

The microbiome of the body has also been linked to inflammation. The bacteria in the gut are responsible for suppressing inflammatory responses to food, so that ingestion of safe substances doesn't disrupt tissues. In contrast, the gut is also crucial in triggering inflammatory responses to consumption of food allergens. Changes in gut bacteria have been correlated to diseases such as Crohn's disease, colon cancer, Alzheimer's, and rheumatoid arthritis, and a host of other diseases (Buford, 2017). Cui et al. (2017) reported that the types of bacteria in the gut can be correlated to coronary heart disease (CHD). It was found that subjects with CHD had lower levels of Bacteroidetes than those who did not have CHD. In addition, those with CHD had higher amounts of Firmicutes than those without CHD. These same trends of gut bacteria are associated with obesity. Obesity is related to deterioration of cardiovascular health. However, in the study, body mass was factored out, in order to isolate the effects of gut bacteria on CHD. Even after this adjustment, CHD was still correlated with changes in gut bacteria biodiversity. (Cui et al., 2017). Since gut bacteria correlate with disease progression in humans, it is important that microbiome of the gut be studied carefully, so that information can be used to improve the health of humans in the future.

## **Methods**

**All procedures were approved by the Bowling Green State University Institutional Animal Care and Use Committee prior to any data collection.**

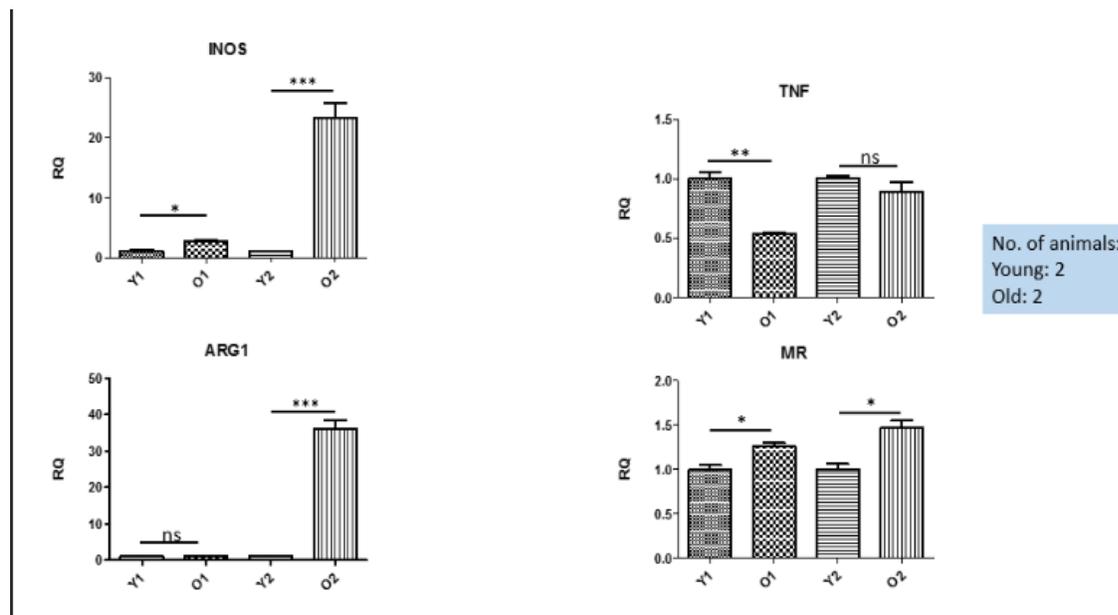
The study involved three mice aged 10 weeks (*dbdb* mice), and two mice aged 10 months (middle aged). The 10 month old mice were representative of the results of aging in the factors studied. They were labelled O1 and O2. The 10 week old mice served as a younger comparison to the older mice,

and were named Y1, Y2, and Y3. The mice were fed a normal chow diet *ad libitum*. Three out of five of the mice were visibly obese, with the O1 mouse being the exception, being smaller and not obese.

The mice were housed in the Animal Sciences Lab at BGSU until data collection started. They were then transported to the University of Toledo Medical Center, euthanized in the lab at UTMC, and certain tissues were collected and processed, including liver and bone marrow. The bone derived macrophages were grown for seven days before testing for their polarity using the markers of inflammation. Fecal samples were also collected over 5 minutes before sacrifice so that the gut microbiota status of each mouse could be determined by 16RNA analysis.

## Results

Figure 1



The young mice were found to have lower levels of iNOS, compared to the O2 mouse, with respective values of about 1.0 and over 20. However, the levels of iNOS in the O1 mouse (not obese) was just slightly higher than the Y1 and Y2.

TNF markers were found to be slightly higher in the Y1 and Y2, as compared to O2 and O1 at respective values of 1.0, around 0.8, and 0.5. O1 had the lowest amount of TNF markers, followed by the young mice.

ARG1 was highest in the O2 mouse, with a relative quantity of nearly 40, and the relative quantities were about the same level in the Y1, Y2, and O2 mice, with relative quantities just above zero.

For MR, the Y1 and Y2 mice were of similar levels of about 1.0, and the O1 and O2 were of similar levels, of about 1.2 and 1.5.

The liver samples did not get good staining, so no usable results were obtained.

Figure 2

## Microbiota Data

		Total	Y	O
Legend	Taxonomy	%	%	%
	Unassigned:Other	0.0%	0.0%	0.0%
	k_Bacteria:p_Actinobacteria	0.0%	0.0%	0.0%
	k_Bacteria:p_Bacteroidetes	34.7%	46.5%	22.8%
	k_Bacteria:p_Cyanobacteria	0.0%	0.0%	0.0%
	k_Bacteria:p_Firmicutes	63.2%	49.5%	76.9%
	k_Bacteria:p_Proteobacteria	0.2%	0.3%	0.0%
	k_Bacteria:p_Tenericutes	0.3%	0.3%	0.2%
	k_Bacteria:p_Verrucomicrobia	1.6%	3.3%	0.0%

No. of animals:  
Young: 3  
Old: 2

In the microbiota for of the young mice, the Bacteroidetes were found in much higher percentages than in the old mice. The Firmicutes were found to be much higher in the old mice than in the young mice.

## **Discussion**

The results from the bone-derived macrophages inflammation markers was inconclusive. The iNOS marker in the O1 was more similar to the Y1 and Y2, while the O2 result was very high compared to the rest of the numbers. Also, the TNF markers were higher in the Y1 and Y2 than in the O1 and O2. The results of the TNF marker were not consistent with previous results, which stated that TNF markers in wounds of aged mice can increase with age (Keylock et al., 2008). In these results, the opposite happened in bone-derived macrophages, which was likely due to the anomalies that were observed in the mice used in the current study. Finally the ARG1 marker was very high in the O2 mouse, and then lower in all the other mice. It was expected that the O1 and O2 mice would present with higher levels of all of these markers, as systemic inflammation tends to increase with age (Wadley et al., 2013). However, in the data collected, it is difficult to report any trends due to low sample size and variability among the older mice. The results were not consistent among the different biomarkers, and were especially inconsistent between the O1 and O2 mice. Therefore, a solid conclusion about inflammation and aging cannot be stated from the results of this experiment.

It is possible that some of the inconsistencies in the results of the inflammation test could be due to the unexpected body composition of the mice studied. The O1 mouse was not obese, and was observed to be of a smaller size than even the young mice with T2D. This was out of the ordinary, as a mouse with T2D is expected to be obese. The O1 mouse's lack of obesity could help to explain why it

had an even lower level of TNF markers than the young mice. This conclusion can be drawn because obesity and inflammation have been reported to be positively correlated (Palmer & Kirkland, 2016).

The O2 mouse was also an anomaly as it was extremely obese, and was believed to have been sick due to poor grooming. This could account for some of the unexpected results observed between O2 and O1. Another explanation for the unclear results would be because only two mice of each age group were studied, due to costs. In the future, the study will be repeated with larger samples of the control and experimental mice, with likely more consistent results.

In the study of fecal samples from the mice, it was found that Firmicutes were much higher in the old mice than in the young. Cui et al. (2017), reported that high levels of Firmicutes were associated with CHD. Also, Buford et al. (2017), stated that the population of Firmicutes has been known to shift in older individuals. Further, the older mice were found to have lower percentages of Bacteroidetes, and the young mice were found to have high levels of Bacteroidetes. This same pattern was observed in a previous study by Cui et al. (2017), who reported that such levels of microbiota were correlated with CHD progression. CHD is a disease that often affects older populations. Therefore, the present study helps to affirm that gut microbiota are influential to the health of an aging individual.

Liver sampling did not yield a readable result. This was likely due to incorrect binding of antibodies to tissue in the liver during staining. In this experiment, it was expected that the antibodies would bind to fat tissues. However, some of the liver sample was degraded, which could have been caused by age, so the antibodies were bound to tissues other than fat tissues. This caused the staining to be unusable for results.

Overall, the test for markers of inflammation did not yield consistent results, so a solid conclusion cannot be derived from the data. However, in studying the microbiota in fecal samples of the mice, it was observed that there is a possible correlation between age and the Firmicutes and

Bacteroidetes species of bacteria. In general, these data point to the conclusion that in older diabetic mice, higher levels of Firmicutes were found, and lower levels of Bacteroides. This can be related back to CHD and inflammation in the body, and shows that gut microbiota could serve as a way to measure the effects of aging and health in the body in the future.

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