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Characterization Of The Metabolic And Cognitive Features Of A Novel Alzheimer'S Disease Rat Model Along With Investigation Of Its Gut Microbiota Change Over Time

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CHARACTERIZATION OF THE METABOLIC AND COGNITIVE FEATURES OF A NOVEL ALZHEIMER'S DISEASE RAT MODEL ALONG WITH INVESTIGATION OF ITS GUT MICROBIOTA CHANGE OVER TIME

by

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A THESIS

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ABSTRACT

Alzheimer's disease (AD) is currently the fifth highest cause of death in the United States and affects primarily individuals of very old age (>80 yrs.). Aging is known to be the biggest risk factor of AD. Recent studies have shown that AD patients have an altered gut microbiota composition. To investigate whether the Alzheimer's disease pathology in the brain could drive gut microbiota changes during the adult and late life stage of the rat, we performed 16S ribosomal RNA sequencing on the fecal samples of the animals at 14 months and 20 months of age. We found a distinctive shift in the gut microbial composition and community structure based off on both genotype and age. The Tgf344-AD rats were also found to have decreased microbiae l diversity compared to controls in middle age and this surprisingly was found to be reversed in advanced age. LEfSe was utilized to assess the differentially represented bacterial taxa between the different genotypes and age groups. Genotypic changes were observed at all levels from phylum to the genus level. Some notable changes were in the genera Bifidobacterium, Ruminococcus, Parasutterella, Lachnoclostridium, Butyricicoccus and Blautia. Aging was found to cause dramatic composition changes. Some of the conserved aging changes in both the Tgf344-AD rats and the control WT rats were decrease in Enterohaldus, Bartonella, Escherichia Shigella and increase in Turibacter, Romboustia, Clostrium_senso_strato. PICRUSt2 was used to determine the differences in functional profile of the gut microbial communities. Our study has demonstrated for the first time, gut microbiome changes in an Alzheimer's disease rat model and aging changes in the F344 rats.

Keywords: Aging, Alzheimer's disease, Metabolism, Microbiome, Gut-brain axis

DEDICATION

This is dedicated to all the researchers (past, present and future) working on studying the biology of aging and aiming to develop intervention strategies to halt and reverse aging processes thereby bringing aging under complete medical control.

“Aging is a disease and it is treatable.” David A Sinclair

“The first 150 year old human is alive right now.” Steven Austad in the year 2000

“Aging is a barbaric, uncivilized phenomenon that shouldn’t really be tolerated in a polite society.” Aubrey de Grey

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
GM	Gut Microbiome
PD	Parkinson's disease
LEfSe	Linear Discriminant Analysis by Effect Size

INTRODUCTION

Aging and Geroscience Hypothesis

Aging is characterized by functional decline at the organismal level combined with the collapse and destruction of independent functioning biological systems in the body. Aging permeates every cell and tissue of the body except the germline. Recently, the major known mechanisms and processes of aging have been highlighted by the Hallmarks of Aging paper in 2013[1]. This showed that aging is a complex multi-factorial process occurring across all tissues and a potential divide-and-conquer strategy may be required to combat all aspects of aging[2]. In the past 2 to 3 decades there have been numerous intervention strategies (genetic, dietary, environmental, pharmacological) that can increase lifespan in mice[3]. Even alterations to singular pathways has shown a dramatic increase in longevity and this shows that with a combinatorial approach targeting all the aging-related damage might have a additive effect. All of the recent studies in model organisms have shown us that lifespan of a species is flexible and it can be easily modulated in short-lived animals and possibly long-lived organisms like human beings.

The Geroscience hypothesis posits that targeting the aging process itself through various therapeutic strategies, will simultaneously delay the onset and occurrence of various aging-related pathologies and diseases and dramatically increase the period of healthy life in an individual's life[4]. Considering that aging is universal to all human beings and especially in older ages during which there is so already so much aging-related damage accumulation to various organs and tissues in the body, targeting aging is crucial for healthy longevity and to reduce the huge economic costs of age-related ill health. Following the Geroscience paradigm of healthcare might be very beneficial and may become ubiquitous especially during the 21st century where the size of the aging population continues to increase rapidly in developed countries[5]. Normal medical practice involves to treat a specific disease after the onset of symptoms and diagnosis usually. This has been successful in the 20th Century by the development of effective treatments for a wide variety of diseases, with tremendous success in infectious diseases. Thereby the life expectancy has increased tremendously. The goal of translational Geroscience is to develop therapies in humans such as those seen in mice which prevent and delay the onset of diseases, thereby extending health span and lifespan[5].

Alzheimer's Disease

Alzheimer's Disease was first studied in modern medicine by Alois Alzheimer in early 1900's[6]. Since then over the course of the 20th century, it's come into prominence as the number of aged people alive has increased a lot with the development of effective treatments for many infectious diseases and other diseases. The next big challenge plaguing human society is that of chronic diseases and other age-related diseases of which aging is the main driver of the disease. This includes several neurodegenerative diseases including Alzheimer's disease. [7] There has been an astounding failure to develop effective treatments for age-related diseases and for AD as evidenced by the failure of numerous clinical trials over the past 20 years [8]

Alzheimer's disease (AD) is the most common form of neurodegenerative disease which affects primarily individuals over 80 years of age. There are 2 variants of the disease seen, Sporadic AD which is seen in individuals over the age of 80 and early-onset AD seen in people below 60 years of age commonly associated with genetic mutations[9]. The current leading theory behind the cause of AD is the "amyloid cascade hypothesis"[10]. This was first put forward in the 1990's after the discovery of genes responsible for early-onset familial AD which included a mutated form of APP protein and the AD-like pathology seen in Down's syndrome patients who have an extra chromosome 21. According to the amyloid cascade hypothesis, AD is caused due to the formation of Beta-amyloid plaques outside the neurons and Neurofibrillary tangles inside the neuron [10]. Unfortunately, all of the drugs targeting the removal of beta-amyloid plaques have failed in human clinical trials even though they succeeded in animal models[11].

This has moved our attention to other characteristics of the disease and disease hypotheses. Clinical studies have shown that obesity and type 1,type 2 diabetes are also risk factors of AD [12]. Type 1 diabetes is when the pancreas is unable to produce insulin due to destruction of the beta cells. Type 2 diabetes is because of insulin resistance in the body. Both result in a high glucose levels in the blood. Some recent studies have shown that insulin deficiency and insulin resistance are mediators of AD pathology. There are some other studies which show that altered peripheral glucose regulation is observed in AD patients, characterized by either insulin resistance [13] or hypoinsulinemia [14]. Further supporting evidence are certain dietary regimens, which reduce diabetes-related physiological changes and have been linked to reduced occurrence of AD in humans. [15] And dietary regimens which tend to enhance the diabetes phenotype have been shown to

worsen AD related lesions in mouse models [16]. There have also been clinical studies showing that AD patients have reduced body weights, some reasons which have been proposed are decreased energy intake or an increase in energy expenditure. Hypermetabolism has also been suggested as a cause for this observation [17]. Hypermetabolism is characterized by an increase in basal metabolic levels. All these point to a metabolic and physiological effect of the disease.

The Gut Microbiome

The human gut microbiome has gained considerable attention in the past 15 years, after many studies which have shown that they may play a causal role in diseases. These studies are a direct result of High Throughput Sequencing (HTS) technology development and its drastic reduction in cost over the past decade [18] . The human gut microbiome contains as much bacterial cells as human cells present in the body, with some estimations going as far as 10 times more bacterial than human cells [19]. Most bacteria residing in the human body are found in the gut. The human GM is established by 3 years of age. The GM has also been found to secrete many biologically active molecules such as short-chain fatty acids (SCFA's) which have diverse functions and still an area of active research. The GM has been associated with many diseases such as Inflammatory Bowel Disease (IBD), ulcers, type 2 diabetes, asthma, neuropsychiatric disorders, obesity, metabolic syndrome etc.; [20]. The GM has been found to play a role in digestion, immunity, metabolism and of considerable interest recently, the gut-brain axis.

The gut-brain axis is a bidirectional communication system which is important for maintaining homeostasis. The microbiota and central nervous system have been found to communicate via endocrine, immune, and neural signaling pathways [21] .There has been a direct link between GM and anxiety, mood disorders and several neurodegenerative diseases. Autism and Parkinson disease (PD) patients have been found to have G.I problems with many PD patients experiencing constipation decades before the onset of motor deficits [22] . There have been several fecal microbiota transplant studies done from human patients with neurodegenerative disease to germ free mice and all the animals have showcased impairment of cognitive function [23]. These studies give support to the hypothesis that gut microbiota plays an etiological role in diseases. Recent studies have also shown that gut microbiota changes are linked with Alzheimer's disease.

There have been 2 studies done to study the gut microbiome changes of AD patients, one at Alzheimer's Disease Research Center (Wisconsin Alzheimer's disease Research Center, USA) and the other at Chongqing Medical University (China), both in the past 4 years [24,25] Both the studies have shown that Alzheimer's disease patients had altered microbial composition with changes at both the Phylum and species level. As of now, there has been no comprehensive survey done on the gut microbial communities of any animal model of AD over their entire lifespan considering both sex differences and age-related changes. Sex differences has been commonly seen in neurodegenerative diseases and is an oft left out variable in studies.

Our Study

In this study we have used Tgf344-AD , a novel Alzheimer's disease rat model first generated in 2013 [26]. There have not been many rat models of AD compared to mice models, with only 3 in total. Rats are better models of the disease when compared to mice, they are much closer to humans in an evolutionary timescale and have a well-developed central nervous system compared to mice. The Tgf344-AD is the only rodent model which has showcased Neurofibrillary tangles, a prominent hallmark of the disease without the addition of other human tau proteins. This could be because rats have 6 isoforms of tau, like that of humans. We hypothesize the Tgf344-AD rat's phenotype to faithfully recapitulate familial AD patient's metabolic and physiological state over the course and progression of the disease, as well as any currently used animal model could do. The is because the driver of the neuropathogenesis are the mutated genes and cerebral amyloidosis is touted play a role in the subsequent metabolic dysregulation. Unlike in sporadic AD patients, where the aging is primary mediator of the disease, and the specific interplay of events and the mechanism which lead to neuropathology has not yet been established even after several decades of research.

INSIGHTS INTO THE GUT MICROBIOTA CHANGES ASSOCIATED WITH AGING AND ALZHEIMER'S DISEASE FROM THE TgF344-AD RAT MODEL

by

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ABSTRACT

Alzheimer's disease (AD) is currently the third highest cause of death in the United States and affects primarily individuals of very old age (>80 yrs.). Aging is known to be the biggest risk factor of AD. Recent studies have shown that AD patients have an altered gut microbiota composition. To investigate whether the Alzheimer's disease pathology in the brain could drive gut microbiota changes during the adult and late life stage of the rat, we performed 16S ribosomal RNA sequencing on the fecal samples of the animals at 14 months and 20 months of age. We found a distinctive shift in the gut microbial composition and community structure based off on both genotype and age. The Tgf344-AD rats were also found to have decreased microbial diversity compared to controls in middle age and this surprisingly was found to be reversed in advanced age. LEfSe was utilized to assess the differentially represented bacterial taxa between the different genotypes and age groups. Genotypic changes was observed at all levels from phylum to the genus level. Some notable changes were in the genera Bifidobacterium, Ruminococcus, Parasutterella, Lachnoclostridium, Butyricicoccus and Blautia. Aging was found to cause dramatic composition changes. Some of the conserved aging changes in both the Tgf344-AD rats and the control WT rats were decrease in Enterohaldus, Bartonella, Escherichia Shigella and increase in Turibacter, Romboustia, Clostrium_senso_strato. PICRUSt2 was used to determine the differences in functional profile of the gut microbial communities. Our study has demonstrated for the first time, gut microbiome changes in an Alzheimer's disease rat model and aging changes in the F344 rats.

Key words Alzheimer's disease, Gut microbiome, Gut-brain axis, Aging

INTRODUCTION

The gut microbiome has been found to be altered in patients with neurodegenerative diseases such as Alzheimer's disease[1], multiple sclerosis[2], Parkinson's disease[3] and psychiatric disorders such as depression, anxiety and autism spectrum disorder[4]. The gut brain axis is a well-coordinated network between the intestinal microbiota, enteric nervous system (ENS) and the central nervous system (CNS) and they communicate with each other through neural, endocrine and immune mediators [5]. Understanding more about the gut microbiota changes associated with neurodegeneration and aging may help us in elucidating the complex crosstalk between the gut and the brain.

Alzheimer's disease (AD) is the most common form of neurodegenerative disease which affects primarily individuals of old age. Aging is the greatest risk factor for Alzheimer's disease and chances of developing dementia or AD increases exponentially in the later stages of life (>65years) [6]. Aging is also hypothesized to be a driver of the disease due to the exclusive nature of the disease to develop in aged brains. Both Alzheimer's disease and the aging process have shown to cause gut microbiome alterations [1,7]. Recent studies in human AD patients from 2 different geographical locations have also shown that gut microbiota changes are linked with Alzheimer's disease [8].

The gut microbiota changes has not only been seen in humans but in animal-models of the diseases too. In vivo studies in mice have shown the gut microbiota to play a causal role in P.D (Parkinson's Disease), as fecal matter transplant of P.D patient's microbiome can initiate cognitive deficits in mice.[3] There is also some evidence that gut microbiome can drive Autism Spectrum Disorder (ASD)[4]. A study in an AD mice model has also shown that gut microbiota alterations are present in those mice[9]. Two recent fecal matter transplant (FMT) studies in AD mice models have shown that it can improve memory and reduce beta amyloid pathology in the brain [10,11].There was also an one-off observational report of dramatic reduction in Alzheimer's disease symptoms in a patient after fecal matter transplantation to treat *C.difficile* infection[12]. These studies posit a view that gut microbiome modulation could be a potential therapy for treating AD.

There has been a tremendous interest in aging research of late especially as the burden of a looming aging population is beginning to be felt. It is estimated that 1 in 6 people will be over the age of 65 by the year 2050 and it will have a tremendous economic burden on countries to care for the increasing number of unhealthy aged people[13]. The Geroscience hypothesis posits that aging process plays a role in many or all chronic diseases and targeting the hallmarks of aging instead of any single disease in a pharmacological way can lead to an extension of healthy lifespan[14]. It provides far greater value and increase in overall quality of life than by targeting a single disease. The gut microbiota has been shown to modulate the lifespan in short-lived vertebrates and a progeroid mouse model. This was demonstrated by fecal matter transplantation studies of young microbiome in

vizzini fish[15] and progeroid mice[16] which resulted in an extension of lifespan. This has shown that the studying the aging microbiome is vital and like other biological systems the microbiome also deteriorates with age. Preventing aging-associated deterioration of the microbiome may be required for healthy aging[17] and possibly rejuvenating the gut microbiome may lead to an prolongation of healthy life in humans similar to what is observed from killifish and mice studies.

Here we have characterized the gut microbiota changes occurring due to the progression and exacerbation of Alzheimer's pathology along with aging associated changes in a novel Alzheimer's disease rat model (Tgf344-AD[18]). We used male Tgf344-AD rats and wildtype control rats for the study. The rats were aged till about 24 months of age and fecal samples were collected at 14 and 20 months of age. 16S rRNA amplicon sequencing was performed on the collected fecal samples after DNA extraction and PCR amplification on the V4 region using Illumina MiSeq instrument. The microbiome data analysis was performed using the most up-to-date techniques, the denoising algorithm DADA2[19] was used to cluster sequences at 99% similarity and taxonomic classification of the ASV's was performed using trained classifiers based on the SILVA[20] database. PICRUSt2[21] was used to predict the functional properties of the gut microbial communities.

Results

Data analysis overview

We were firstly interested in looking at i. the genotypic differences at middle age (14months) and old age (20months) and ii. the age-related changes for both the WT control rats and the Tgf344-AD rats. There was a total of 30 samples sequenced from Tgf344-AD rats and control WT rats at 2 different time points. All of the samples combined had a total frequency of 2,713,807 amplicon reads which were sequenced. Each sample had a mean of 90,460 reads with a maximum of 127,750 and a minimum of 46,564 reads per sample. After denoising with DADA2, the total frequency of features were 1,615,899 with 1547 unique features. The average number of features per sample was 53,863.3 and with the maximum and minimum features per sample were 83,951 and 29,316. Taxonomic Classification of the ASV's was performed done using the SILVA database[20]. The FASTQ files have been submitted to SRA ID number:

Richness and Diversity of experimental groups

Firstly, to assess the richness and diversity of the samples, alpha diversity was calculated. Faith's phylogenetic diversity and Observed ASV's were the 2 metrices calculated and comparisons were done to assess both the genotypic changes and age-related changes. The 14-month-old Tgf-344AD rats showed a decrease in both metrics, faiths PD and observed ASV's (Fig 1, p-value 0.0483 and 0.017). At the 20-month time point, this was surprisingly found to be reversed. The WT control rats showed a steep decrease in both

alpha diversity metrices at 20 months of age. At the 20-month time point, there was significantly increased diversity in the Tgf344-AD rats when compared to WT control rats (Fig1, p-value 0.0428 and 0.038). The age-related decrease in both diversity and number of observed ASV's for the WT animals was significant (Fig1, p-value 0.0025 and 0.0037) while for the AD rats there is a tendency towards an increase, but it was not statistically significant. There is some evidence with higher alpha diversity being linked with better health and lower alpha diversity being linked with various disease states[22].
The Firmicutes: Bacteroidetes (F:B) ratio has been viewed as a biomarker of intestinal homeostasis and frequently observed with obesity. The Tgf344-AD rats had a slightly increased Firmicutes:Bacteriodetes ratio when compared to WT control rats but did not reach statistical significance. There was no differences at 20 months of age.

Changes in community structure

To assess the similarity or dissimilarity of the overall microbial communities, Beta diversity indices was calculated using the Unweighted Unifrac and Jaccard distance metrics. Principal Coordinates analysis carried out using the calculated Unweighted Unifrac and Jaccard distance matrix which demonstrated a striking change in the microbiota community structure between middle aged and old age animals. There was also a slight clustering based on genotype observed for 14 months group but it was not as distinct as those separated by age. Permutational multivariate analysis of variance (PERMANOVA) was carried out on the distance between groups to analyze if the variation was of statistical significance. The age- related changes was significant for both the WT group (Unweighted Unifrac: p-value 0.004; Jaccard: p-value 0.024) and the AD group (Jaccard: p-value 0.049; Unweighted Unifrac did not reach statistical significance p-value 0.083). The genotypic changes were not found to be significant although at 20 months it came close. (Jaccard distance p-value 0.076).

Overall composition changes due to Alzheimer's phenotype and aging

Next, we looked at whether there were any overall composition changes to the gut microbial communities due to Alzheimer's phenotype and aging. The gut microbial composition are represented through bar plots of relative abundance at taxonomic levels: Phylum, Class, Order. At the Phylum level, the most abundant taxa are Firmicutes, Bacteroidetes, Desulfobacterota, Verrucomicrobiota, Actinobacteriota, Proteobacteria and Cyanobacteria (Fig 3). There were genotypic changes in phyla observed at 14 months of age (Bacteriodetes reduced for AD group, Fig 3) but no genotypic changes were seen at 20 months of age. The WT age-related change showed an decrease of Proteobacteria and the AD age-related change showed an increase of Actinobacteria Fig 3 (e).
At the Class level, the most abundant taxa are Clostridia, Bacilli, Bacteroidia, Desulfovibrionia, Verrucomicrobiae, Actinobacteria, Vampirivibrionia (Fig). At 14 months of age, Class Bacteriodia, Actinobacteria were reduced in AD group (Fig 4(a)). While at 20 months of age, Class Gammaproteobacteria was found to be increased in the

AD group (Fig 4(c)). The WT age-related changes showed an decrease of Coriobacteria and Gammaproteobacteria while the AD group showed an decrease of Coriobacteria and increase in Actinobacteria (Fig 4(a)(c)) .
At the Order level, the most abundant taxa are Oscillospirales, Lachnospirales, Bacteroidales, Erysipelotrichales, Lactobacillales, Clostridiales. At 14 months of age, Orders Bacteroidales, Clostridia and Bifidobacteriales were reduced in the AD group while Burkholderiales was increased in AD group at 20 months of age. At the Order level the conserved aging changes between WT and AD groups were Coriobacteriales, Rhizobales and Enterobacterales which decreased, while Erysopelotrichales, Clostridales and Pepstreptococca Tissierellaes which increased (Fig 4(a),(c)) .

Differentially represented bacterial taxa
To determine the changes in the gut microbial communities occurring due to Alzheimer's disease phenotype and aging, LEfSe (Linear Discriminant Analysis by Effect Size) was utilized. In general, the aging driven changes were much more prominent than changes due to Alzheimer's disease pathology.

The family level genotypic changes as determined by LEfSe were Bifidobacteriaceae, Prevotellaceae, Hungateiclostridaceae, Sutterellaceae which were all decreased at 14 months and Butyricicoccaceae, UCG_010 and Sutterellaceae increased at 20 months. At the genus level, there was a decrease in Ruminococcus, Bifidobacterium, Parasutterella and Prevotellaceae and increase in Streptococcus and Lachnoclostridium at 14 months. The major change at 20 months was genus Blautia. The conserved aging changes at the genus level between WT and AD groups are Enterohaldus, Bartonella and Escherichia shigella which were decreased while Turicibacter, Clostridium_sensu_stricto_1, Romboustia increased.

Discussion

The gut microbiota has been associated in many diseases including neurodegenerative diseases. The gut brain axis is hypothesized to have a major role in the disease progression and possibly may even play an etiological role. Aging also influences the microbiome diversity, richness, and composition dramatically[23]. In this study, we demonstrated for the first time, altered gut microbiome changes in a novel Alzheimer's disease rat model, and the dramatic changes and shifts in microbiome composition and structure due to aging in the Fischer 344 rat model. This transgenic rat model is considered one of the best preclinical models to study AD since it recapitulates full spectrum of AD pathology better than the mice models of the disease. One notable feature is that we are looking at changes in the later stage of the animal's life, as Alzheimer's disease is an aging-associated disease and is seen only in people afflicted by aging during the later stages of life. We have characterized the gut microbiota changes at a stage in the animal's life (late adult to old age) similar to when it affects humans.

There is strong evidence from human clinical studies showing that there are gut microbiome alterations seen in a plethora of diseases[24]. Studies done in germ-free mice and antibiotic-treated mice have consolidated this view that the microbiome plays an important role in these diseases and microbiome composition must be maintained for a healthy life and healthy aging. Fecal matter transplantation studies have shown that many beneficial characteristics of the donor phenotype such as exercise[25], caloric restriction[26–28] can be recapitulated in the recipient thereby showcasing the important role the microbiome plays in maintaining homeostasis. The opposite is also true where transplant from donor with natural or diet-induced obesity[28] (in the case of mice) or various diseases can also recapitulate some of that negative phenotype.

Numerous previous studies have shown that microbial diversity and richness to go down with age[17]. This was observed with the normal aging process, but with centenarians the opposite was found to be true. The diversity was increased compared with younger people and this was found to be the case in numerous populations of centenarians in different geographical locations. Centenarians are a prime example of healthy aging as they escape many of the debilitating effects of chronic diseases. Diversity was also found to have a negative correlation with frailty[29]. Similar to human Alzheimer's patients[1], alpha diversity was found to be decreased in our Tgf344-AD rats at 14 months of age but not 20 months where aging might have obscured the changes. As expected with aging, the alpha diversity was again found to decrease with age in our rats. The dramatic change in the community structure due to aging in our rats was also similar to other rodent microbiome aging studies. Beta diversity changes due to genotype were less prominent when compared with aging which might show that the changes due to AD pathology might be subtle compared to system-wide effects of aging.

Diabetes, obesity increase the chances of getting Alzheimer's disease, and insulin resistance and disrupted glucose metabolism have been hypothesized to be causal factors in Alzheimer's disease. We have previously demonstrated that peripheral glucose metabolism and insulin sensitivity are disrupted in Tgf344-AD rats because of the AD phenotype (cite Hemant's paper). Firmicutes:Bacteriodetes ratio is a commonly studied variable in microbiome studies and a certain ratio has been associated with the maintenance of normal intestinal homeostasis. Increase or decrease in the Firmicutes:Bacteriodetes ratio has been found to be observed with several disease states.
In our study there were no significant differences found between groups although at 14 months of age the AD group had a higher F:B ratio although it did not reach significance. There was also no age-related changes in the ratio although gut dysbiosis has been hypothesized to occur with aging. There have been 2 previous microbiome studies done in human Alzheimer's disease patients so far and they have shown the diversity to be decreased and alterations in community structure like our Tgf344-AD rats. However there has been differences in the results observed with respect to differentially expressed

taxa, the phyla Bacteroidetes increased and Actinobacteria decreased in one study while the opposite was observed in the other study. This discrepancy may have been due to many other factors such as geographical location, diet, ethnicity etc., Further studies investigating whether SCFA (short chain fatty acids) levels are changed in bloodstream and the intestines are required along with characterizing these changes. This is because altered SCFA production might play a possible mechanistic role in disease initiation as something similar was observed in the case of Parkinson's disease where SCFA treatment could reproduce many features of the disease in mice. In our Tgf344-AD rats, the phyla Bacteroidetes was found to be decreased similar to one of the human studies. Other studies have also found Bacteroides fragilis to be reduced in patients with cognitive impairment and brain amyloidosis.

Another notable change was the reduction of Bifidobacterium in the Tgf344-AD rats which is a well-known probiotic genus. Bifidobacterium has been linked with anti-inflammatory properties and decrease in intestinal permeability. A previous study has also demonstrated that Bifidobacterium supplementation can impact cognition in an anxious mouse strain. This change in Bifidobacterium was not visible at 20 months of age where aging might have obscured the genotypic changes. In our Tgf344-AD rats, Parasutterella which has been previously associated with irritable bowel syndrome[30] was found to be reduced at 14 months of age. A study found that increasing Parasutterella levels were correlated with decreasing low-density lipoprotein levels[31]. Ruminococcus is a genus which has been linked with Alzheimer's disease in two previous studies and in our Tgf344-AD rats, they showed a similar decrease[32]. Prevotellaceae has been previously associated with APOE positive humans and transgenic mice[33], and in our Tgf344-AD rats they were found to be decreased.

Blautia which has been linked with Alzheimer's from clinical studies, were also found increased in the Tgf344-AD rats at 20 months of age[1] It has also been previously associated in Parkinson's disease and multiple sclerosis patients. Blautia has been found to have an inverse association with visceral fat[34]. Blautia has been found to be involved in anti-inflammatory processes. Higher Blautia has been associated with disturbances in glucose metabolism, type-2 diabetes and high fat diet. It was also found to be depleted in obese children and implicated in intestinal inflammation[35]. The butyrate producer, Butyricicoccus was also increased in the Tgf344-AD rats at 20 months of age.

One interesting observation was that a lot of taxa changes were observed in the family Lachnospiracea in the Tgf344-AD rats when compared to control rats. Similar changes have been reported in human Alzheimer's microbiome studies too. This may indicate a possible common phenomenon occuring because of the Alzheimer's disease pathology. Lachnospiraceae are main producers of SCFA's including butyrate and form an important part of the gut microbiota.

Aging conserved changes common to both the Tgf344-AD rats and the control WT rats were Turicibacter, Romboutsia and Clostridium_sensu_stricto increased and Enterohaldus, Bartonella and Escherichia shigella decreased. Turicibacter has been previously found to be increased with aging in other studies[36,37] and it is also correlated with higher fat mass[38]. Romboutsia has been termed a harmful bacteria with increases observed in certain disease states[39]. Changes in the family level due to aging such as Erysipelotrichaceae, Peptostreptococcaceae were also found to be changed in other aging microbiome studies in mice[40,41]. No changes were detected for *A.municiphilia* between the Tgf344-AD rats and WT control rats. *A.municiphilia* has been previously associated with obesity, type-2 diabetes.

There have been previous reports of gut microbiota changes in Alzheimer's disease mice models. But the Tgf344-AD rat model is seen as a better Alzheimer's model due to its ability to develop the full spectrum of AD pathology including the neurofibrillary tangles and frank neuronal loss. So the use of the Tgf344-AD rat model would be a better rodent model to study the microbiome changes compared to other models. There is evidence that gut microbiota might enhance the AD pathology as seen by an microbiome depletion study in the APP/PS1 mice model done using antibiotics[42].Another thing to consider is the microbiome composition differences between rats and humans and that any common changes occurring might be obscured. But rodent models can be useful for mechanistic studies and intervention studies for the microbiome modulation therapies for the treatment of Alzheimer's disease.

An unanswered question is whether changes in gut microbiome composition alone can initiate some features of the disease such as cognitive deficits or anxiety. This was previously observed with Parkinson's disease transplant studies in germ-free mice. Future directions in studying the gut microbiota of the Tgf344-AD rats would be to check the microbiome composition of the female rats and compare the sexes, as sex-specific differences are observed for this disease. Also, longitudinal human studies in Alzheimer's disease patients are also required to better understand the changes. The current studies performed have been cross-sectional in nature.

Another thing to consider would be the period of onset of gut microbiota changes in the Tgf344-AD rats and whether it precedes behavioral and metabolic changes or comes after it. Animals models of the disease can be useful to dissect many changes occuring due to the AD pathology observed in the brain and compare it through the prism of aging. It is also important to recognize that the disease may be fundamentally different in sporadic cases of AD which occurs in humans without any genetic defect and is the most common variant of the disease. The animals models of the disease can be considered faithful models of familial AD seen in humans. In summary, we found gut microbiota changes in the Tgf344-AD rats when compared to age-matched control WT rats with taxonomic

changes observed at various levels. The aging-associated changes in the gut microbiome was prominent and shows the important role aging plays in disrupting homeostasis in the body and gut permeability. There were also some common changes seen when compared to human clinical microbiome studies in AD patients.

Methods

Animals

One pair of TgF344-AD rats were obtained from Dr. Terrence Town of USC and these were bred to produce an initial litter. After maturation, these rats were bred to wildtype rats on a Fisher 344 background to produce the experimental colony. All rats were maintained in a fixed light−dark cycle with ad libitum access to food and water. All animals were housed at Research Support Building (RSB) University of Alabama, Birmingham. All experiments were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC).

Fecal sample collection

Fecal pellets were collected from the Tgf344-AD and F-344 control rats at ages: 8-9 month, 14 months and 20 months of age. Only Male rat samples were chosen for this study. The fecal pellets were collected at the same time of the day for consistency. 2-3 fecal pellets were collected per rat. The fecal pellets were collected with sterilized forceps and place into 1.5mL tubes and immediately stored in ice. All the collected fecal pellets were transported to the -80C freezer within 2 hours of collection. Autoclaved cages for each individual rat was used during the fecal sample collection. Before the DNA extraction process, the fecal pellets were transported to a different location with a -20C freezer.

DNA Extraction and 16s rRNA Sequencing

DNA extraction process was performed on the chosen samples using the Fecal DNA Isolation Kit from Zymo Research (cat. no. D6010) according to the manufacturer's instructions. The isolated DNA was then used for PCR or stored in Tris-EDTA buffer for later use. The DNA was quantified in a Spectrophotometer before PCR. PCR amplification of the V4 region of the 16S rRNA gene was carried out using unique barcoded primers to create an amplicon library.
The primer information is provided below:

Forward primer
5'AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMG
CCGCGGTAA-3'

Reverse primer
5’CAAGAGAAGACGGCATACGAGATNNNNNNAGTCAGTCAGCCGGACTACHV
GGGTWTCTAAT-3’

The individual PCR products were run on an Agarose gel, bands visualized by UV illumination and they were in turn excised and purified by QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). The PCR products were sequenced using the Illumina MiSeq platform by 250bp paired-end sequencing.

Bioinformatics and statistical analysis
Demultiplexed data files was obtained from the UAB Microbiome Resource after the sequencing process. In the first step of the analysis, the FASTQC files were imported into the Quantitative insights for microbial ecology 2 (QIIME2) environment as .qza files using the “import” function and the manifest method. Check quality
DADA2 denoising algorithm was used to cluster the sequence at 99% similarity. DADA2 workflow implements the following steps: filtering of the reads, dereplication, chimera removal and merging of paired-end reads. The final output from DADA2 is an ASV (Amplicon Sequence Variant) table and the representative sequences file containing the ASV ID matching with a amplicon sequence read. A Phylogenetic tree was constructed using the q2-phylogeny plugin and the align-to-tree-mafft-fasttree pipeline. For all statistical analysis and whenever pairwise comparisons were made, only planned comparisons were done. Posthoc analysis was not used for group comparisons. The planned comparisons looked at differences occuring due to i. genotype at 14 months of age and 20 months of age, and ii. age-related changes between WT groups and AD groups.

For diversity analysis, rarefaction was performed at a 14,000 reads sampling depth to ensure the same number of random sequencing reads were used for all samples. Faith’s phylogenetic diversity and Observed ASV’s were the two alpha diversity measures calculated. A non-parametric ANOVA was used to compare the alpha diversity measures. Unweighted Unifrac and Jaccard distance were the two Beta diversity metrics calculated. Unweighted Unifrac matrix and Jaccard distance matrix were used to create the Principal Coordinate Analysis (PCoA) plot in R using phyloseq. Statistical analysis was performed using the Unweighted Unifrac and Jaccard distances between groups using Permutational multivariate analysis of variance (PERMANOVA) testing. The taxonomic composition bar plots at different levels of classification was made using the QIIME2 plugin using the SILVA taxonomy output file. Linear discriminant analysis by effect size (LEfSe) was used to determine the differentially abundant taxa between the different groups. LEfSe uses a non-parametric factorial Kruskal Wallis test to detect features which are differentially represented between groups. The results were plotted as a Cladogram and as LDA histograms. As with other analyses, both genotypic and age-related comparisons were made.

Microbial function prediction

The functional profile of the gut microbial communities was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) pipeline. The output ASV feature table and representative sequences files from DADA2 were used as input for PICRUSt2. The ASV abundances were normalized using the 16S rRNA gene copy numbers using the Integrated Microbial Genomes (IMG) database. E.C. ID's (Enzyme Classification) and KO ID's (Kyoto Encyclopedia of Genes and Genomes-KEGG) were predicted. The statistical analysis was performed using STAMP software. The data was also visualized using STAMP.

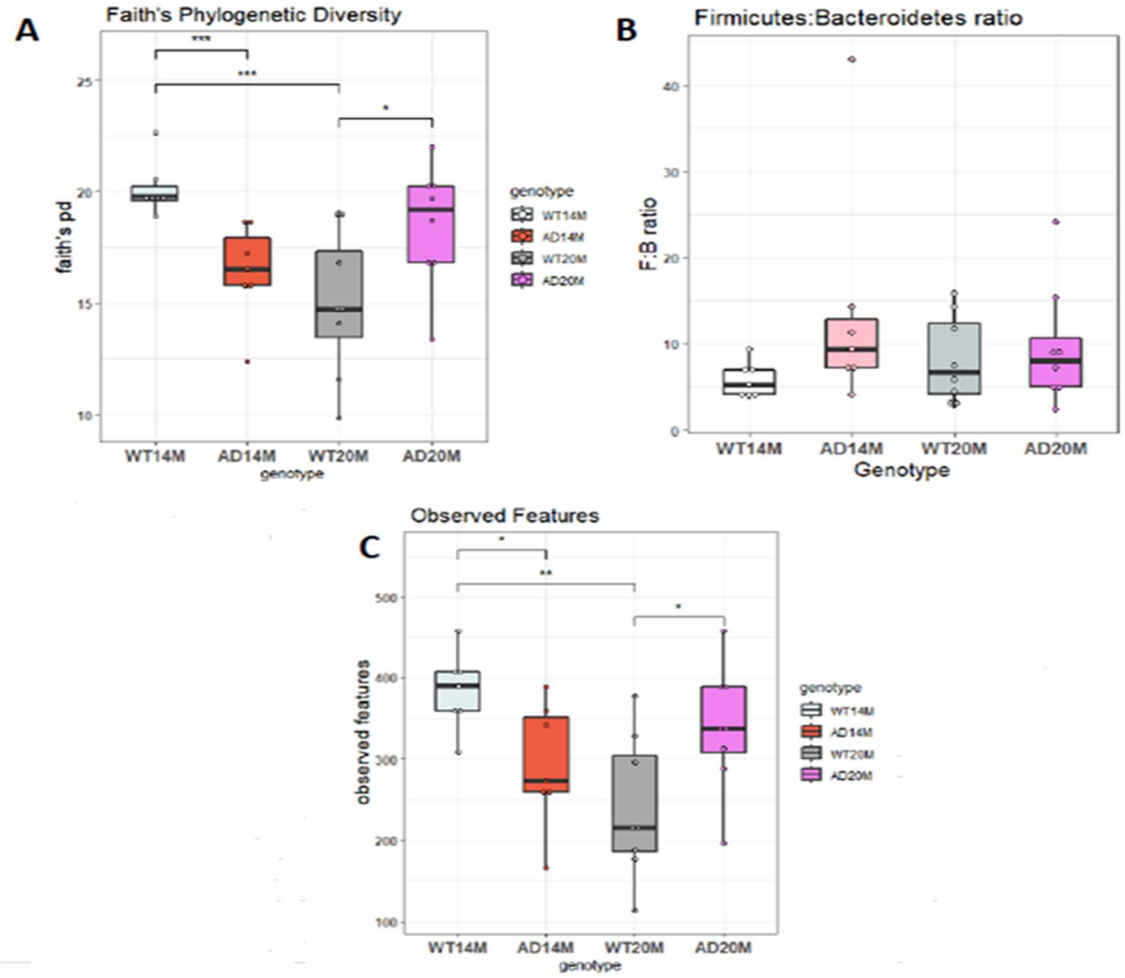
REFERENCES

1. Vogt, N. M. et al. Gut microbiome alterations in Alzheimer's disease. Sci Rep 7, 13537 (2017).
2. Berer, K. et al. Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. Proc Natl Acad Sci USA 114, 10719–10724 (2017).
3. Sampson, T. R. et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell 167, 1469-1480.e12 (2016).
4. Sharon, G. et al. Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. Cell 177, 1600-1618.e17 (2019).
5. Cox, L. M. & Weiner, H. L. Microbiota Signaling Pathways that Influence Neurologic Disease. Neurotherapeutics 15, 135–145 (2018).
6. Corrada, M. M., Brookmeyer, R., Paganini-Hill, A., Berlau, D. & Kawas, C. H. Dementia incidence continues to increase with age in the oldest old: The 90+ study. Ann Neurol. 67, 114–121 (2010).
7. Galkin, F. et al. Human Gut Microbiome Aging Clock Based on Taxonomic Profiling and Deep Learning. iScience 23, 101199 (2020).
8. Zhuang, Z.-Q. et al. Gut Microbiota is Altered in Patients with Alzheimer's Disease. JAD 63, 1337–1346 (2018).
9. Zhang, L. et al. Altered Gut Microbiota in a Mouse Model of Alzheimer's Disease. JAD 60, 1241–1257 (2017).
10. Holsinger, R. M. D. & Elangovan, S. Neuroprotective effects of fecal microbiota transplantation in a mouse model of Alzheimer's disease: Development of new models and analysis methods/validation of pre-clinical methods. Alzheimer's & Dementia 16, (2020).
11. Sun, J. et al. Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. Transl Psychiatry 9, 189 (2019).
12. Hazan, S. Rapid improvement in Alzheimer's disease symptoms following fecal microbiota transplantation: a case report. J Int Med Res 48, 030006052092593 (2020).
13. An Aging Nation: The Older Population in the United States. 28.
14. Austad, S. N. The Geroscience Hypothesis: Is It Possible to Change the Rate of Aging? in Advances in Geroscience (eds. Sierra, F. & Kohanski, R.) 1–36 (Springer International Publishing, 2016). doi:10.1007/978-3-319-23246-1_1.
15. Smith, P. et al. Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. eLife 6, e27014 (2017).

16. Bárcena, C. et al. Healthspan and lifespan extension by fecal microbiota transplantation into progeroid mice. Nat Med 25, 1234–1242 (2019).
17. Kumar, M., Babaei, P., Ji, B. & Nielsen, J. Human gut microbiota and healthy aging: Recent developments and future prospective. NHA 4, 3–16 (2016).
18. Cohen, R. M. et al. A Transgenic Alzheimer Rat with Plaques, Tau Pathology, Behavioral Impairment, Oligomeric A , and Frank Neuronal Loss. Journal of Neuroscience 33, 6245–6256 (2013).
19. Callahan, B. J. et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13, 581–583 (2016).
20. Yilmaz, P. et al. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucl. Acids Res. 42, D643–D648 (2014).
21. Douglas, G. M. et al. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol 38, 685–688 (2020).
22. MetaHIT consortium et al. Richness of human gut microbiome correlates with metabolic markers. Nature 500, 541–546 (2013).
23. Kong, F., Deng, F., Li, Y. & Zhao, J. Identification of gut microbiome signatures associated with longevity provides a promising modulation target for healthy aging. Gut Microbes 10, 210–215 (2019).
24. Bull, M. J. & Plummer, N. T. Part 1: The Human Gut Microbiome in Health and Disease. Integrative Medicine 13, 6 (2014).
25. Lai, Z.-L. et al. Fecal microbiota transplantation confers beneficial metabolic effects of diet and exercise on diet-induced obese mice. Sci Rep 8, 15625 (2018).
26. Fabbiano, S. et al. Functional Gut Microbiota Remodeling Contributes to the Caloric Restriction-Induced Metabolic Improvements. Cell Metabolism 28, 907-921.e7 (2018).
27. Pérez-Matute, P., Íñiguez, M., de Toro, M., Recio-Fernández, E. & Oteo, J. A. Autologous fecal transplantation from a lean state potentiates caloric restriction effects on body weight and adiposity in obese mice. Sci Rep 10, 9388 (2020).
28. Zoll, J. et al. Fecal microbiota transplantation from high caloric-fed donors alters glucose metabolism in recipient mice, independently of adiposity or exercise status. American Journal of Physiology-Endocrinology and Metabolism 319, E203–E216 (2020).
29. Jackson, M. A. et al. Signatures of early frailty in the gut microbiota. Genome Med 8, 8 (2016).
30. Chen, Y.-J. et al. Parasutterella , in association with irritable bowel syndrome and intestinal chronic inflammation: Parasutterella may be related with IBS. Journal of Gastroenterology and Hepatology 33, 1844–1852 (2018).

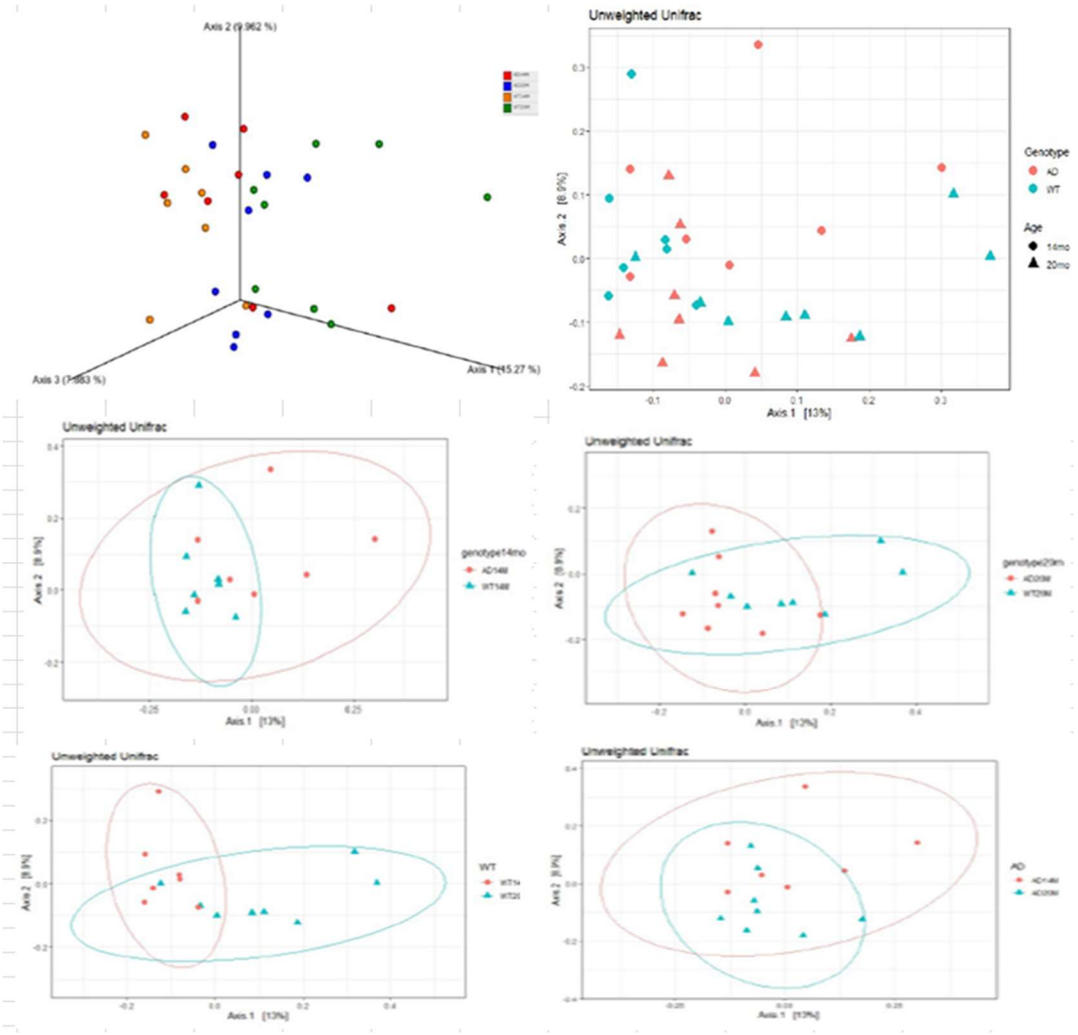
31. Bush, J. R. Increasing levels of Parasutterella in the gut microbiome correlate with improving low-density lipoprotein levels in healthy adults consuming resistant potato starch during a randomised trial. 9 (2020).
32. Liu, P. et al. Altered microbiomes distinguish Alzheimer's disease from amnestic mild cognitive impairment and health in a Chinese cohort. Brain, Behavior, and Immunity 80, 633–643 (2019).
33. Tran, T. T. T. et al. APOE genotype influences the gut microbiome structure and function in humans and mice: relevance for Alzheimer's disease pathophysiology. FASEB j. 33, 8221–8231 (2019).
34. Ozato, N. et al. Blautia genus associated with visceral fat accumulation in adults 20–76 years of age. npj Biofilms Microbiomes 5, 28 (2019).
35. Benítez-Páez, A. et al. Depletion of Blautia Species in the Microbiota of Obese Children Relates to Intestinal Inflammation and Metabolic Phenotype Worsening. mSystems 5, e00857-19, /msystems/5/2/msys.00857-19.atom (2020).
36. Zhang, X. et al. Age-related compositional changes and correlations of gut microbiome, serum metabolome, and immune factor in rats. GeroScience (2020) doi:10.1007/s11357-020-00188-y.
37. Liu, A. et al. Aging Increases the Severity of Colitis and the Related Changes to the Gut Barrier and Gut Microbiota in Humans and Mice. The Journals of Gerontology: Series A 75, 1284–1292 (2020).
38. Binyamin, D. et al. The aging mouse microbiome has obesogenic characteristics. Genome Med 12, 87 (2020).
39. Bojović, K. et al. Gut Microbiota Dysbiosis Associated With Altered Production of Short Chain Fatty Acids in Children With Neurodevelopmental Disorders. Front. Cell. Infect. Microbiol. 10, 223 (2020).
40. Conley, M. N. et al. Aging and serum MCP-1 are associated with gut microbiome composition in a murine model. PeerJ 4, e1854 (2016).
41. Langille, M. G. et al. Microbial shifts in the aging mouse gut. Microbiome 2, 50 (2014).
42. Harach, T. et al. Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. Sci Rep 7, 41802 (2017).

Figure 1. Richness and Diversity of the Gut microbiota



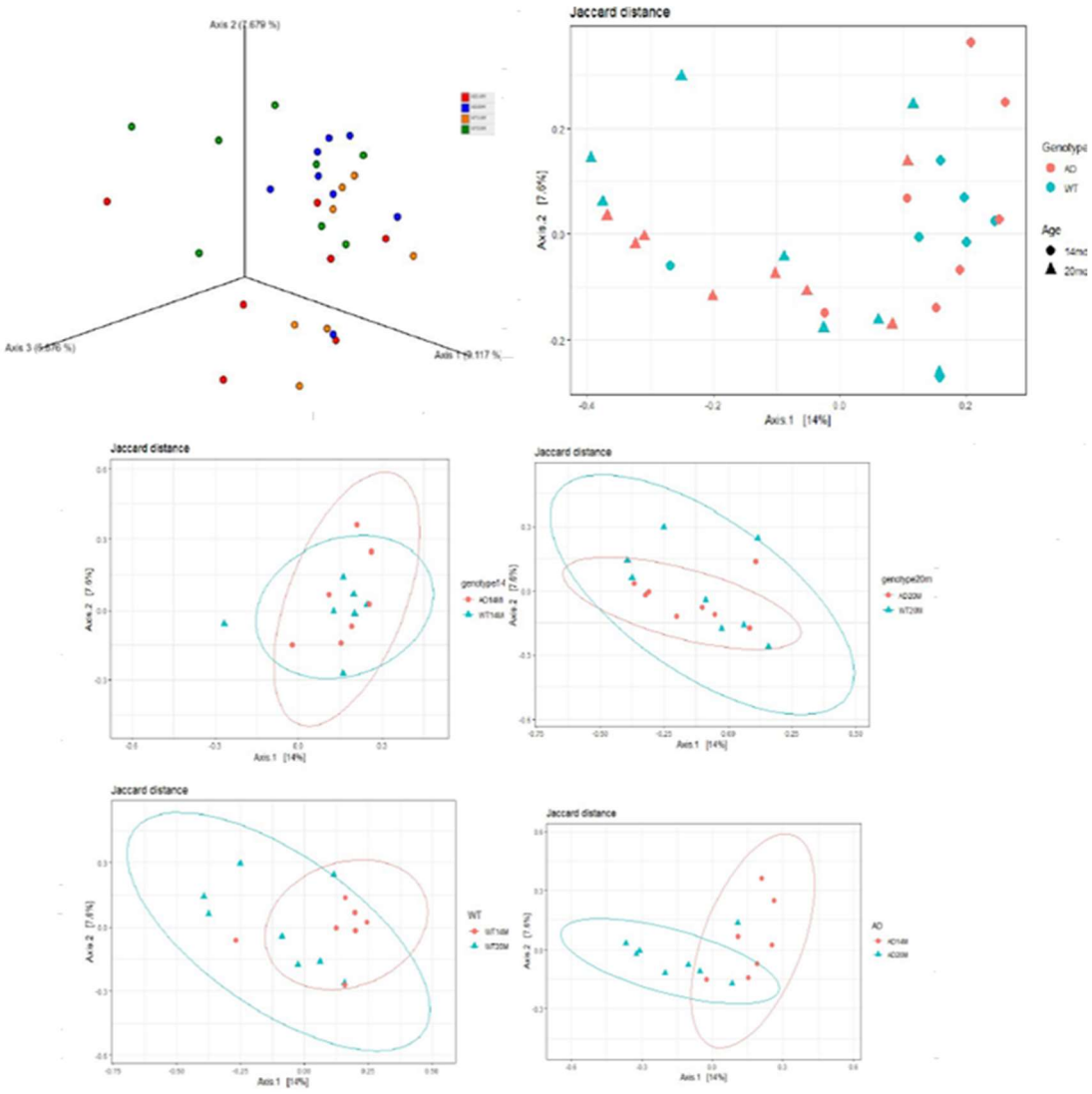
Richness and Diversity of the Gut microbiota across groups. (A) Faith's Phylogenetic diversity calculated for the 4 groups, (B) Observed Features calculated for the 4 groups, (C) Firmicutes: Bacteroidetes ratio was calculated to evaluate intestinal homeostasis and dysbiosis.

Figure 2. Beta Diversity analyses- Unweighted Unifrac



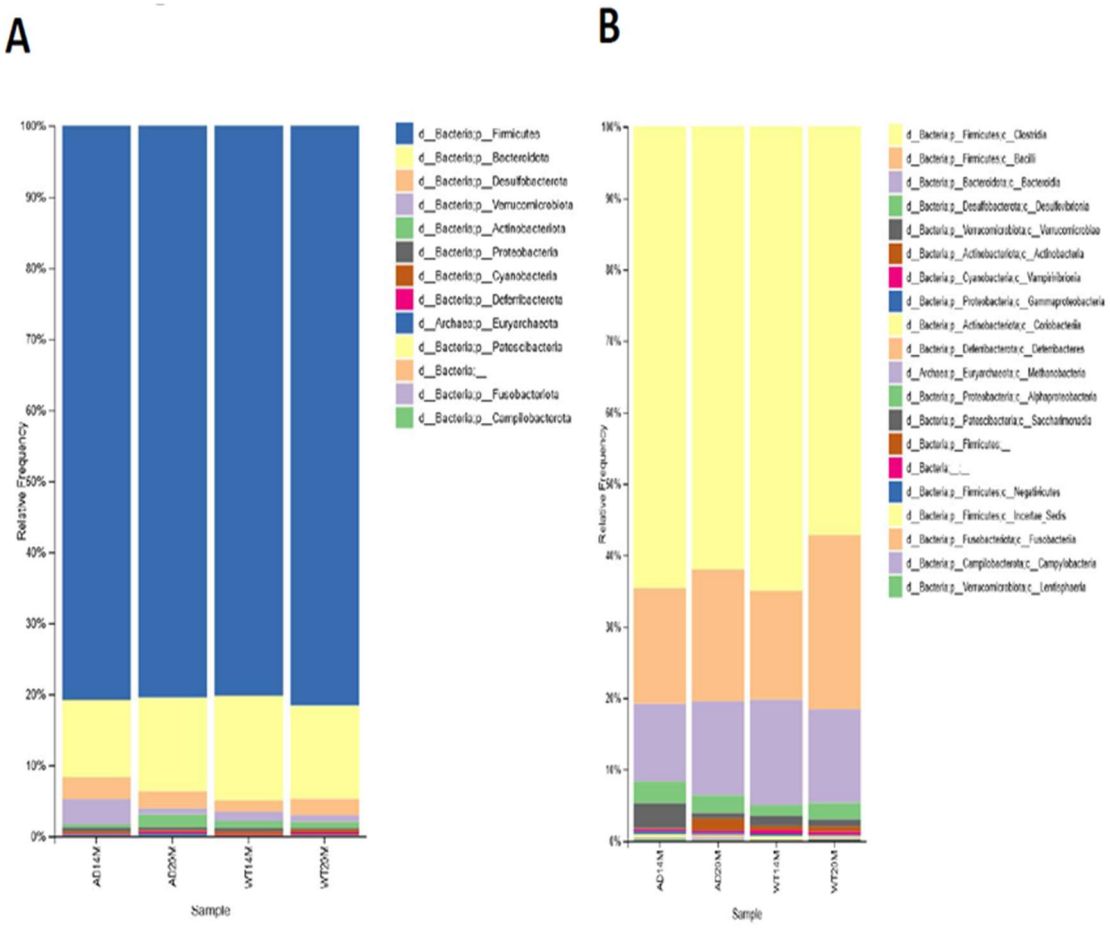
3D Principal coordinates analysis (PCoA) derived from Unweighted Unifrac and Jaccard distances among the 4 groups (PERMANOVA)
(c) 2D PCoA plot of Unweighted Unifrac distance representing all 4 groups, (d) stratified to show aging changes for the WT and AD groups, (e) stratified to show genotypic changes at 14 and 20 months. age.

Figure 3. Beta Diversity analyses- Jaccard Distance



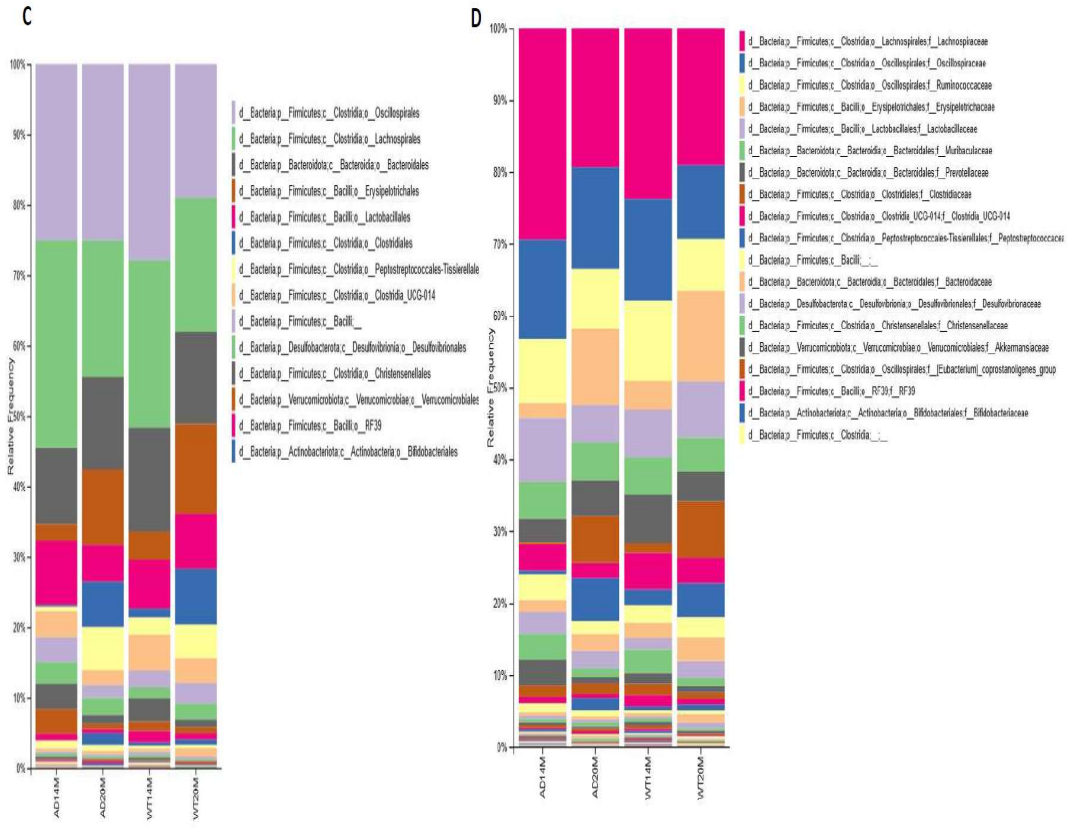
(f) 2D PCoA plot of Jaccard distance representing all 4 groups, (g) stratified to show aging changes for the WT and AD groups, (h) stratified to show genotypic changes at 14 and 20 months.

Figure 4. Composition of gut microbial communities



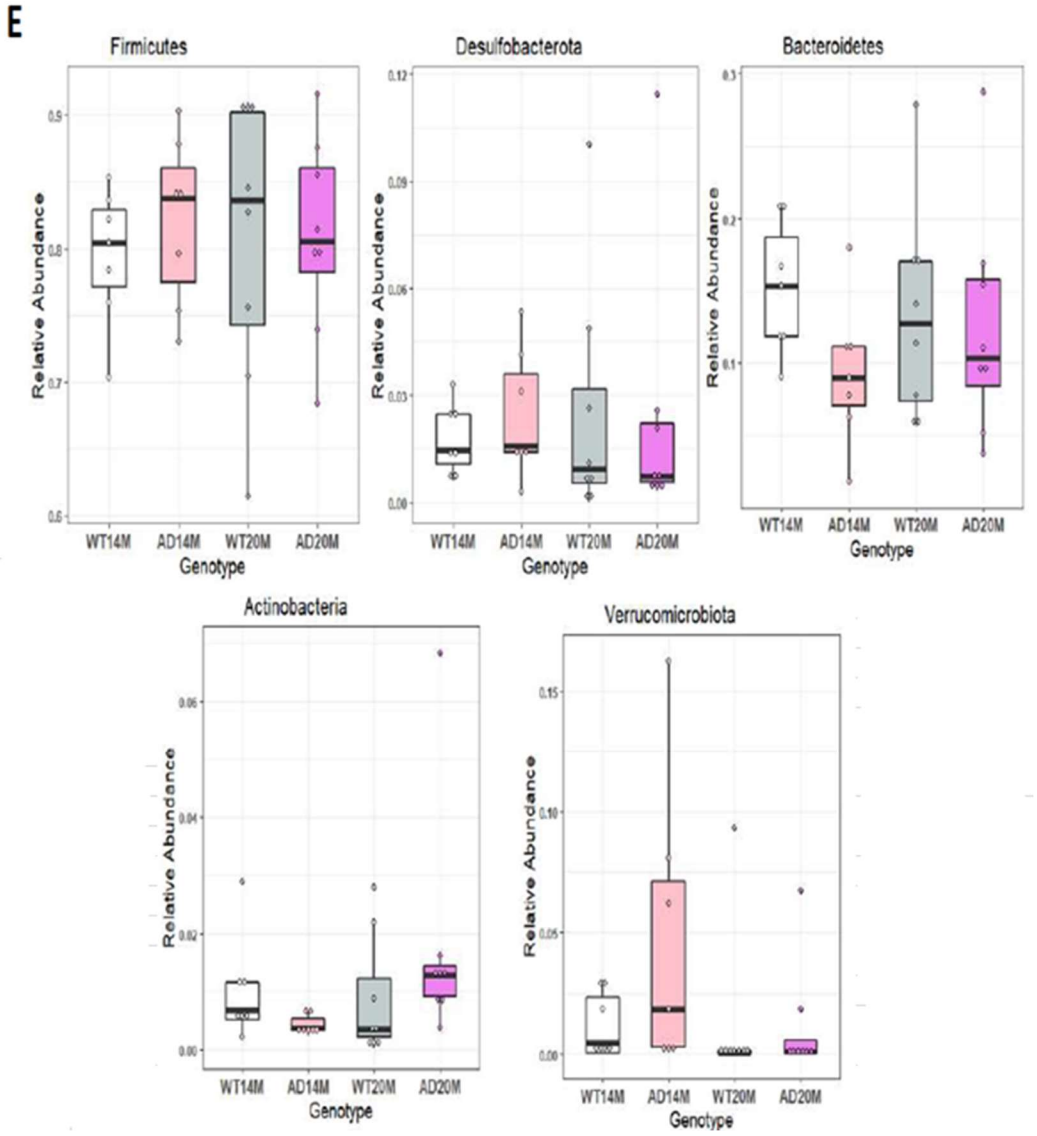
Composition of gut microbial communities of the Tgf344-AD and WT control rats visualized at different Taxonomic levels. (A) Phylum, (B) Class, (C) Order, (D) Family. (E) Box and whisker plots of the top 5 Phyla present in the experimental animals.

Figure 5. Composition of gut microbial communities of rats



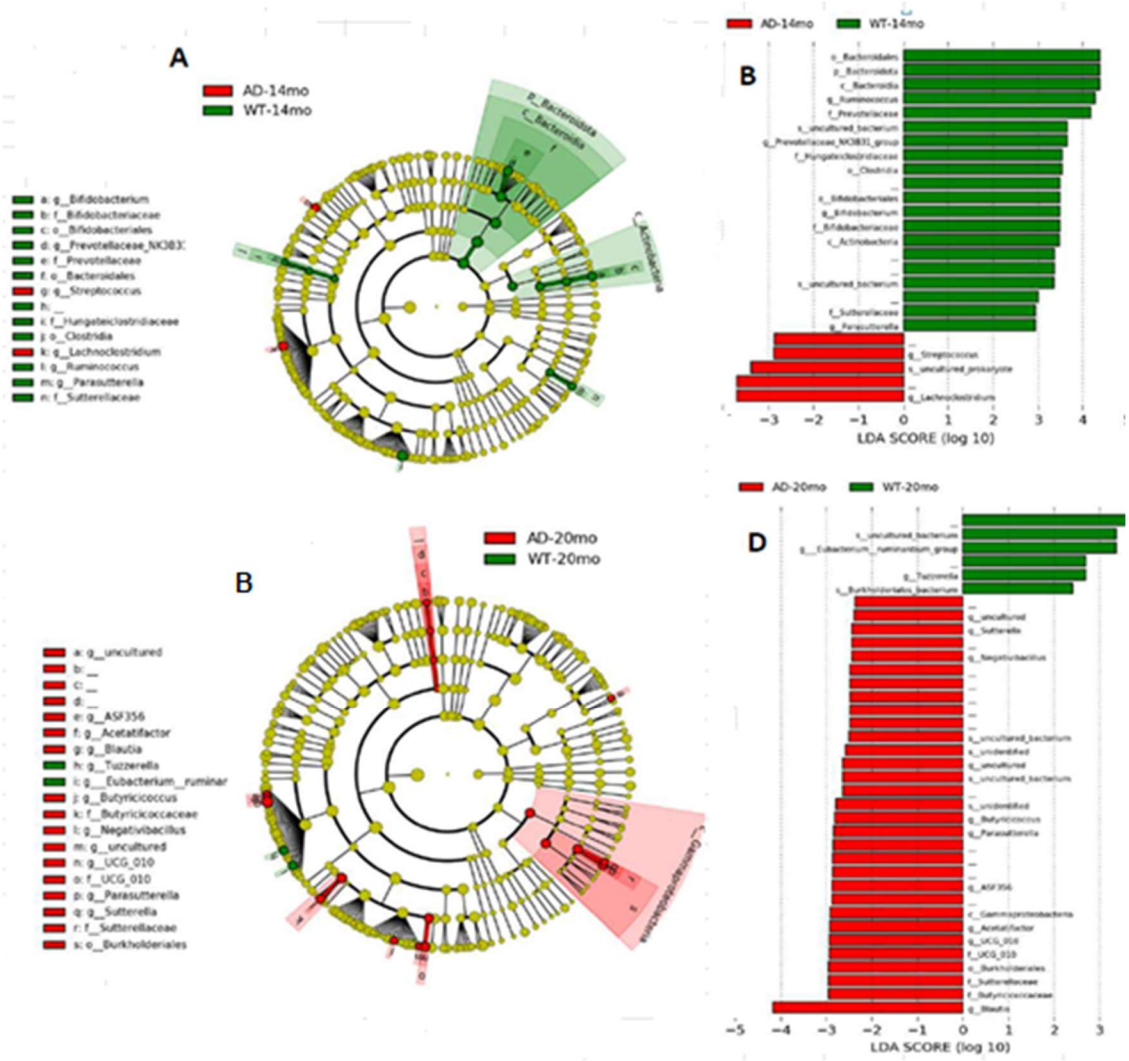
Composition of gut microbial communities of the Tgf344-AD and WT control rats visualized at different Taxonomic levels. (A) Phylum, (B) Class, (C) Order, (D) Family. (E) Box and whisker plots of the top 5 Phyla present in the experimental animals.

Figure 6. Composition of gut microbial communities shown as Box plots



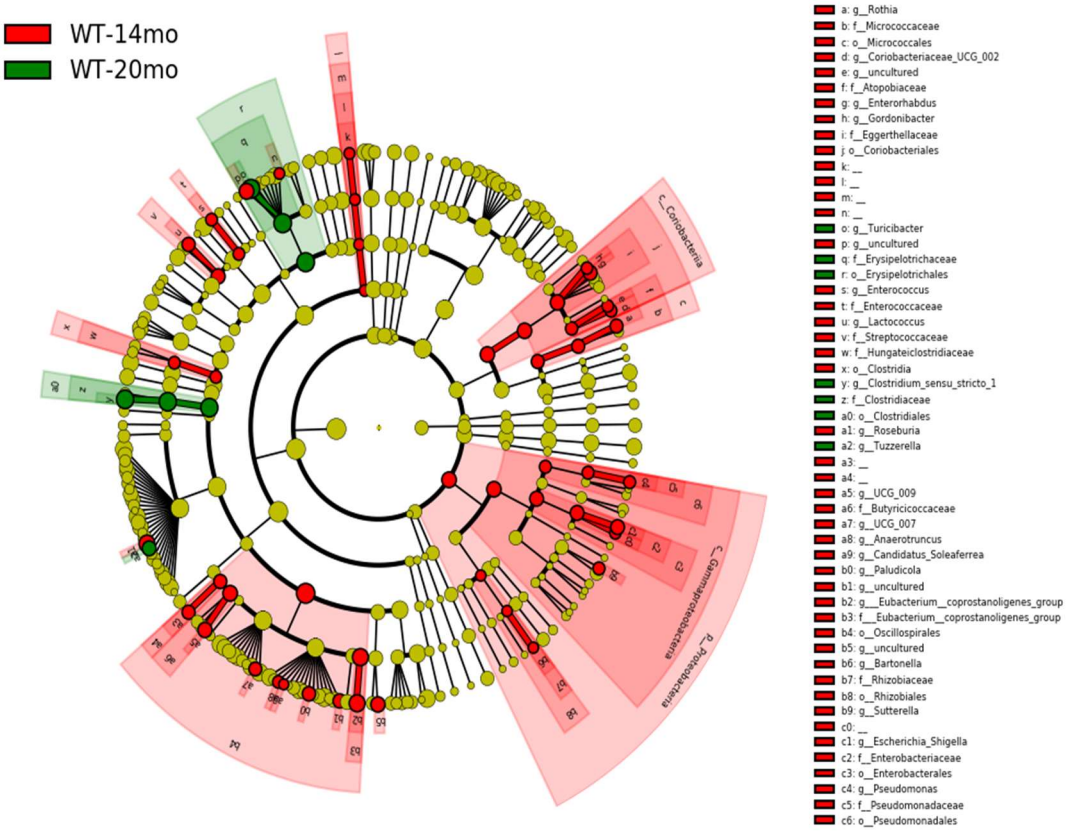
Composition of gut microbial communities of the Tgf344-AD and WT control rats visualized at different Taxonomic levels. (A) Phylum, (B) Class, (C) Order, (D) Family. (E) Box and whisker plots of the top 5 Phyla present in the experimental animals.

Figure 7. The effect of AD phenotype on the gut microbial communities



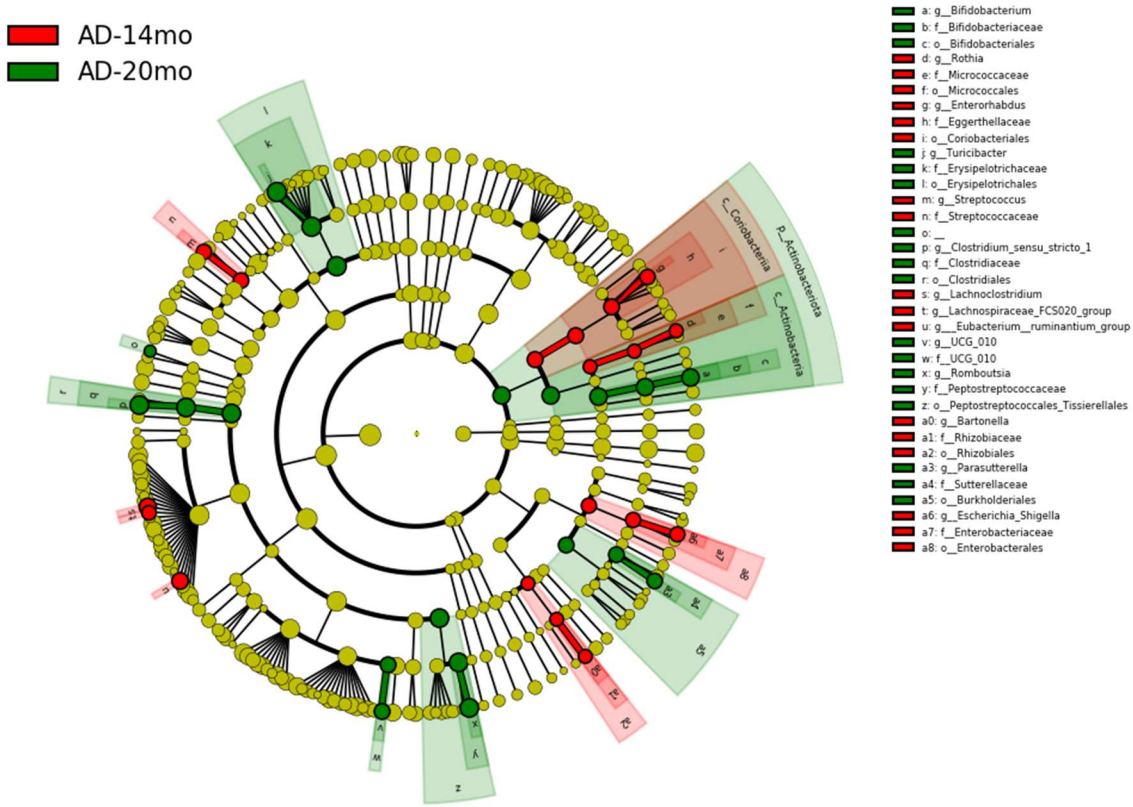
Microbial taxa that were differentially represented was determined by LEfSe and visualized by Cladogram (A), (C) and LDA histograms (B), (D) at 14 months of age and 20 months of age.

Figure 8. The effect of aging on WT animals- Cladogram



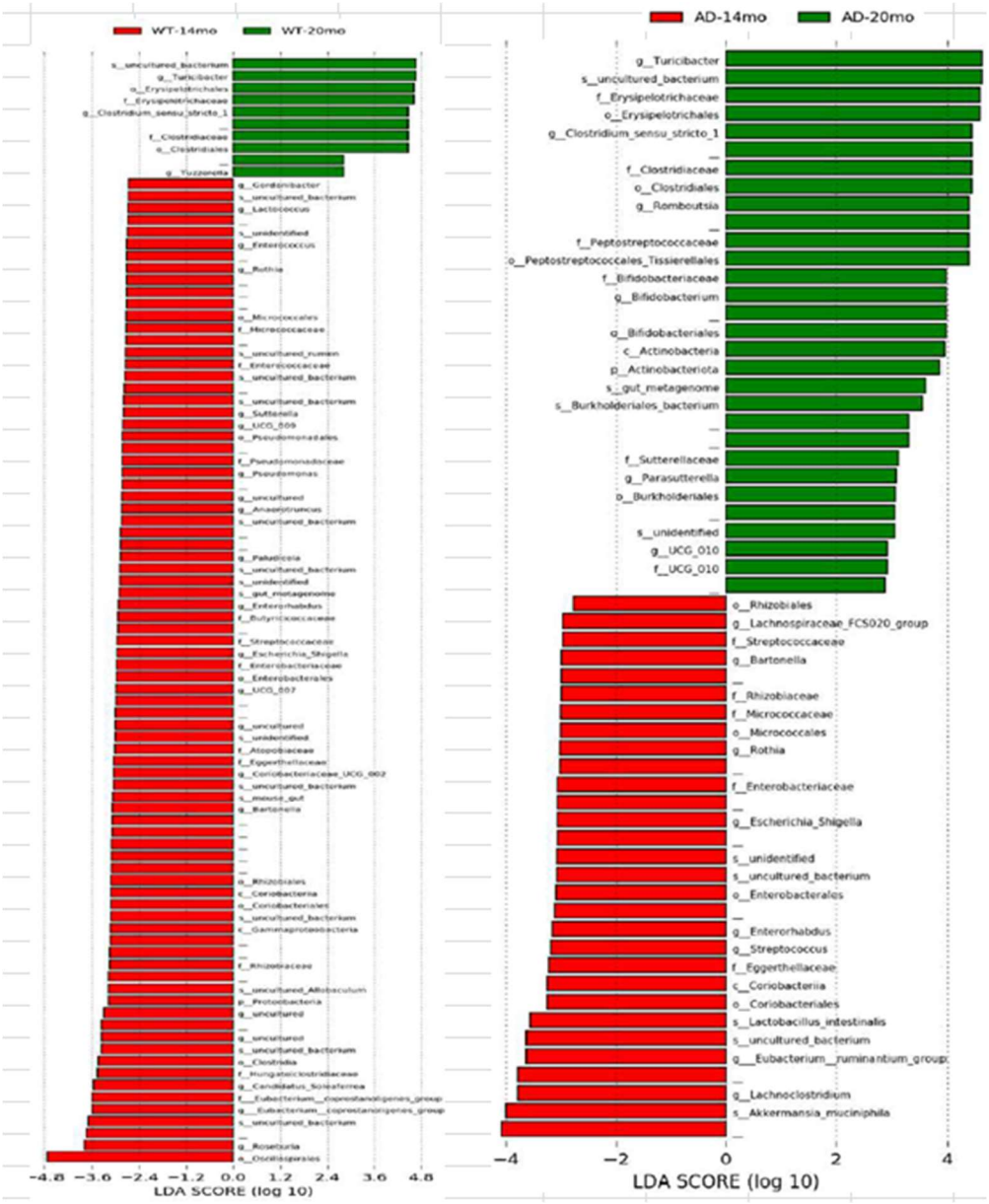
The effect of aging on the gut microbial communities of the Tgf344-AD rats and WT rats. Microbial taxa that were differentially represented was determined by LEfSe and visualized by Cladogram (a), (c) and LDA histograms (b), (d) at 14 months of age and 20 months of age.

Figure 9. The effect of aging on AD animals- Cladogram



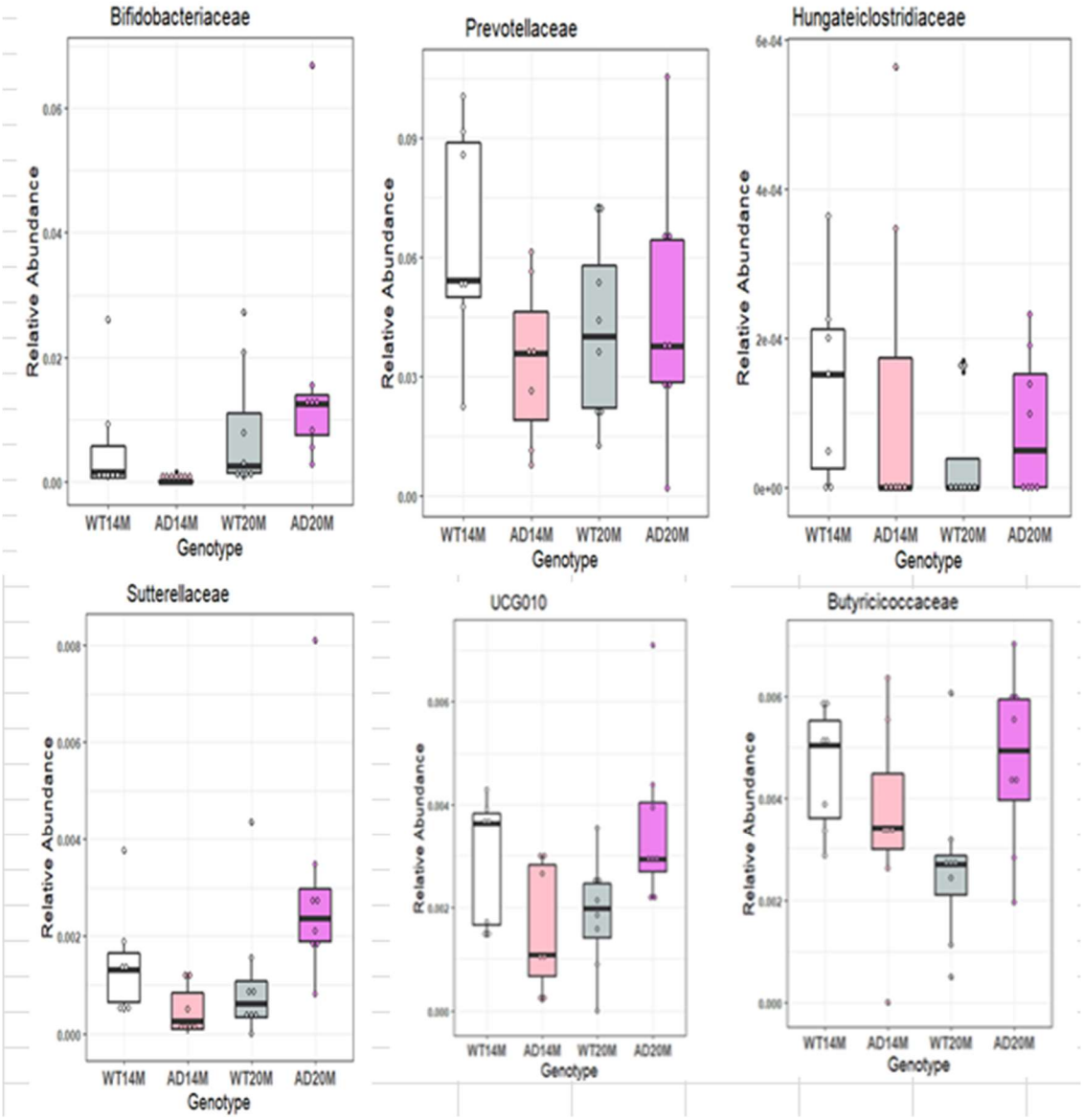
The effect of aging on the gut microbial communities of the Tgf344-AD rats and WT rats. Microbial taxa that were differentially represented was determined by LEfSe and visualized by Cladogram (a), (c) and LDA histograms (b), (d) at 14 months of age and 20 months of age.

Figure 10. The effect of aging on the gut microbial communities- LDA Histogram



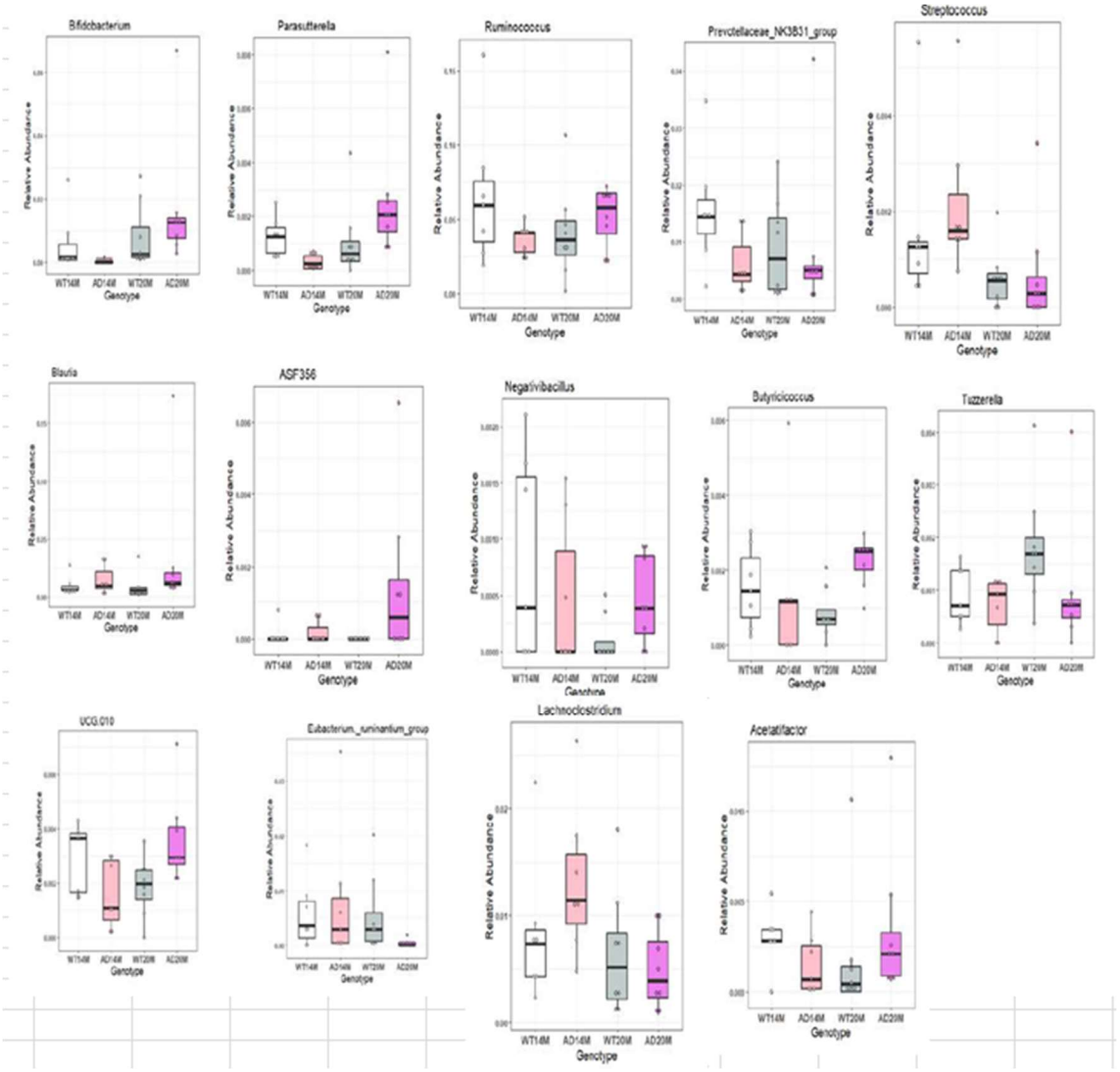
The effect of aging on the gut microbial communities of the Tgf344-AD rats and WT rats. Microbial taxa that were differentially represented was determined by LEfSe and visualized by Cladogram (a), (c) and LDA histograms (b), (d) at 14 months of age and 20 months of age.

Figure 11. Differentially represented taxa due to genotype at the Family level



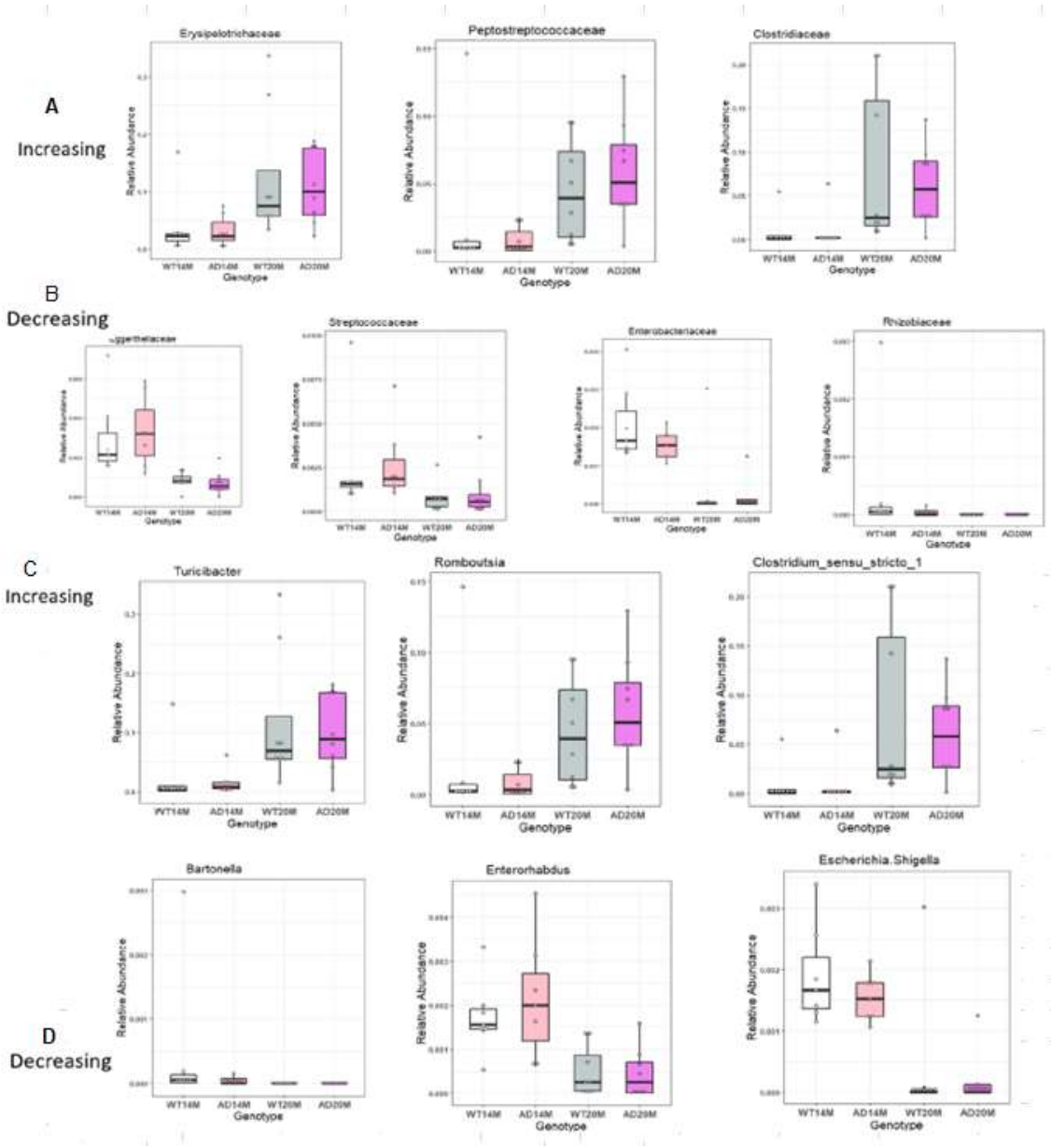
Differentially represented taxa in the Tgf344-AD rats when compared to WT controls at the Family level

Figure 12. Differentially represented taxa due to genotype at the Genus level



Differentially represented taxa in the Tgf344-AD rats when compared to WT controls at the Genus level

Figure 13. Conserved aging-associated changes between WT and AD rats



Conserved aging-associated changes between the AD and WT groups. (a)-(c) Families increasing with age. (d)-(f) Families decreasing with age. (g)-(i) Genera increasing with age, (j)-(l) Genera decreasing with age

CHARACTERIZING THE BEHAVIOURAL AND METABOLIC CHANGES IN THE TGF344 AD RAT DURING EARLY TO MID-ADULTHOOD

by

HEMANT SRIVASTAVA, AKASH NAGARAJAN, LIOU SUN

In preparation for *Neurobiology of Aging*

Format adapted for thesis

ABSTRACT

Alzheimer’s disease (AD) is the most common form of neurodegenerative disease which affects primarily individuals of very old age. It is estimated the number of people with Alzheimer's disease will increase to 13.8 million by the year 2050 due to steady rise in life expectancy and the proportion of old people alive, which has been powered by improvements to healthcare. AD has been previously associated with diabetes and obesity in human clinical studies. Mice models of AD have shown metabolic dysregulation prior to cognitive decline. But it is unknown if this phenomenon is singular to mice or it may be conserved in other species too. We conducted an experiment to test if peripheral metabolism dysregulation occurs in the Tgf344-AD rats and whether it coincides with behavioral changes. At 9 and 12 months of age, a battery of behavioral tests were conducted along with glucose and insulin tolerance tests (GTT and ITT). A distinct sex specific phenotype was observed in peripheral metabolic dysregulation. Male TgF344-AD rats showed no change in insulin sensitivity at 9 and 12 months while female TgF344-AD showed decreased insulin sensitivity. There were also body composition changes observed with no changes in males, but females showed increased body weight and fat mass at 9 and 12 months of age. This gives strong evidence that AD pathology may drive or result in peripheral metabolic changes in glucose and insulin metabolism.

INTRODUCTION

Alzheimer's disease (AD) which is a progressive neurological disease is a rapidly growing public health issue as it is a that according to previous reports from the World Health Organization (WHO) is the fifth leading cause of death in the United States of Americans aged 65 and older. For individuals aged 65, the risk of developing AD doubles every 5 years, which indicates that one in three individuals aged 85 and older will have AD (Ballard et al., 2011). AD is currently an irreversible, progressive brain disease that damages neurons, resulting in the loss of brain function. Early in AD diagnosis, patients will experience onset memory defects, loss, and cognitive impairments that progressively worsen as the disease continues to spread and destroy additional brain tissue and neurons, despite the use of therapeutics (Castellani, Rolston, & Smith, 2010; Hyman et al., 2012).

Current research outlines the Amyloid Cascade hypothesis(Kocahan & Dogan, 2017), which is the leading theory behind how Alzheimer's disease progresses. This hypothesis postulates that improper degradation of amyloid precursor protein (APP) occurs due to usage of β -secretase, which induces γ-secretase to cut the APP molecule in such a way that B-amyloid peptides form. These peptides clump together and lead to deposition of neurotoxic amyloid beta plaques. Additionally, tau protein gets hyperphosphorylated, thus inducing the formation of neurofibrillary tangles, which further contributes to AD symptoms. However, though previous studies have extensively worked with the Amyloid Cascade hypothesis to develop treatments for AD, there has been a repetitive failure to develop effective therapeutics for AD(Kumar, Singh, & Ekavali, 2015; Mehta, Jackson, Paul, Shi, & Sabbagh, 2017).

Over the past decade there has been a recent surge in literature suggesting neurodegeneration in AD is also linked to metabolic differences and insulin dysregulation (Kang, Lee, & Lee, 2017). Studies have shown that Type-2 diabetes (T2D) affected adults have a 50%-150% increased risk of developing AD vs the general population (Biessels, Staekenborg, Brunner, Brayne, & Scheltens, 2006); additionally, AD has been associated with insulin and IGF-1 dysregulation in the brain (Steen et al., 2005), and chemically disrupted insulin/IGF-1 signaling in neuronal tissue is sufficient to create the pathological features of AD (Lester-Coll et al., 2006). This has prompted some researchers to consider AD as type 3 diabetes.(de la Monte & Wands, 2008).

From previous research, we know that the pathophysiological dysfunctions of AD arise before the appearance of cognitive deficits (Sperling, Mormino, & Johnson, 2014). Thus, in our study, we aimed to characterize the metabolic profile of a preclinical model of AD during early to middle adulthood. We hypothesized that insulin dysregulation and metabolic impairments associated with a pre-diabetic phenotype occur before the onset of the cognitive symptoms of AD. For our study, we used a preclinical AD model

established in 2013: the TgF344-AD rat. These rats were created by co-injecting pronuclei with "Swedish" mutant human APP (APPsw) and Δ exon 9 mutant human presenilin-1 (PS1ΔE9) onto a Fisher344 background (Cohen et al., 2013). These rats are the first AD model to show age-dependent tau hyperphosphorylation, neuronal loss, and cognitive impairments including decline in memory without the introduction of additional human transgenes unrelated to AD (Berkowitz, Harvey, Drake, Thompson, & Clark, 2018; Do Carmo & Cuello, 2013; Munoz-Moreno, Tudela, Lopez-Gil, & Soria, 2018).

A previous study using APP/PS1 mice on a C57BL/6J background found transgenic mice predisposed to AD showed significant impairments in glucose metabolism and a negative response to the introduction of exogenous insulin compared to their WT counterparts at ages as early as 2 months (Macklin et al., 2017). However, no studies have been done assessing the metabolic features including insulin sensitivity paralleled with the neuropathology of AD in this model.

Materials and Methods

Animals

All procedures used in this experiment have been approved by the National Institute of Health Office of Animal Care and Use and followed the guidelines set by the Institutional Animal Care and Use Committee. A pair of TgF344-AD$^{+/-}$ breeders (wild type females and heterozygous males) were obtained from Dr. Terrence Town of USC and these were bred to produce heterozygous and control male and female offspring. Tail snips were obtained at weaning and were used to genotype the animals as previously described (Cohen et al., 2013). The rats were group housed in a facility that maintained a normal 12-h light/dark cycle with ad libitum access to food and water.

Glucose and Insulin tolerance tests

Rats were fasted overnight for 20 hours before they underwent GTT. The fasted rats were administered a intraperitoneal injection of glucose of 1g/kg body weight. Blood glucose levels were measured at different time points- 0,14,30,45, 60 and 120 minutes. Rats were fasted for 4hrs before ITT was done by intraperitoneal injection of 7 IU porcine insulin (Sigma-Aldrich, St.Louis, MO) per kg of BW. Blood glucose levels were measured at different time points- 0,14,30,45,60 and 120 minutes. A two-sample t-test was done to compare specific timepoints for statistical significance.

Body Composition Analysis with Quantitative Magnetic Resonance (QMR)

Body composition was determined using the EchoMRI™ Whole Body Composition Analyzer for rats in conjunction with the EchoMRI 2018 Body Composition Analyzer software. System tests were run each day that animals body compositions were analyzed. Rats were placed in the holding tube which was then placed horizontally into the machine. The animals were scanned using primary accumulation of 1 and measuring total

water mass in addition to fat and lean mass. All measurements were done at the UAB Small Animal Phenotyping Core.

Behavioral Analyses

Prior to conducting behavioral analyses, animals were habituated to handling for 14 days. Additionally, all animals were given an hour to habituate to the testing room in their home cages. Behavioral assays were conducted using the facilities provided by the UAB Behavioral Assessment Core. The behavioral assays were all conducted in a 2-week period, with elevated plus maze and dual object recognition tests in the first week and the Morris Water Maze test in the second week. All movement in the behavioral analyses was tracked with a camera driven tracker system, i.e., Ethovision 14 (Noldus, The Netherlands). The system recorded the position of the animal in the arena at 15 frames/second.

Elevated Plus Maze

The Elevated Plus Maze (EPM) was used to specifically measure anxiety. The maze consisted of four arms. Each arm was 60 cm long and 10 cm wide. The 4 arms were located 60 cm above the ground. Two of the arms had 60 cm high opaque walls surrounding them while the other two arms had no walls. At the beginning of each trial, the animals were placed in the center of the arena and were allowed to move around freely for a 4-minute period. After each trial, the apparatus was wiped down with chlorhexidine and 70% ethanol.

Dual Object Recognition Test

The Dual Object Recognition test was used to assess learning and memory and was performed on two consecutive days. The maze consisted of an opaque, square Plexiglass chamber measuring 90 cm x 90 cm x 40 cm. On the first day, there were two identical small objects strategically placed in the test chamber. However, on day two, one of the objects was switched out so that there were then two distinct objects located in the testing chamber. On each test day, the animal was placed into the testing chamber with the objects and allowed to explore for a period of 10 minutes. After each trial, the apparatus was wiped down with chlorhexidine and 70% ethanol.

Morris Water Maze

The Morris Water Maze Test was used to assess spatial learning and long-term memory and it took 5 days. The apparatus we used was a blue plastic pool that is 183 cm in diameter and was filled up with water. In the pool, we had a see-through platform that was 10 cm in diameter and 0.5 cm underneath the surface of the water and always in the Northwestern quadrant of the tank. Over the first 4 days, each rat did four two-minute trials to find the platform from each of the four cardinal points of the tank. Each trial was stopped once the rat was on the platform for a 5 second period. If the rats were unable to find the platform after 2 minutes, they were guided to the platform. Finally, on day 5, in

addition to doing the normal 4 trials beginning from each of the cardinal points in the tank, an additional trial was conducted. This was called the probe trail and in it, the platform was removed, and the rats were placed into the tank for a 1-minute time period to freely swim around while being tracked. After each trial, any fecal samples left in the pool were removed and the water was changed out daily.

Statistical Analysis

GraphPad Prism 7 or R was used for all statistical analyses. Differences between groups were evaluated by t-test, one-, or two-way ANOVA. Post-hoc pairwise comparisons were conducted using Tukey's or Sidak's post-hoc tests as appropriate. Differences were considered significant at $p<0.05$. All data are represented as mean ± SEM or mean ± pooled SEM.

Results

Behavioral Tests

To establish the behavioral changes at 9 and 12 months of age, we performed a suite of behavioral assays—the Elevated Plus Maze, the Morris Water Maze, and the Dual Object Recognition Tests.

Elevated Plus Maze

We used the Elevated Plus Maze (EPM) to establish when the onset of anxiety-like behaviors occurred. At 9 months of age, both male AD rats ($p = 0.0112$) and female AD rats ($p = 0.0478$) spent less time in the EPM when compared to WT rats of the same age and sex. (Fig. 1A). Additionally, 9-month-old male AD rats entered the open arms of the EPM less frequently than age-matched WT controls ($p < 0.0001$) while no significant difference was detected between female AD rats and age-matched WT controls ($p = 0.1450$) (Fig. 1B). At 12 months of age, both male AD rats ($p = 0.0083$) and female AD rats (0.0119) again spent less time in the open arms of the EPM compared to age-matched WT controls (Fig. 1C). At 12 months of age, male AD rats again showed a lower frequency of entering the open arms of the EPM ($p < 0.0001$) compared to age-matched WT controls. At 12 months of age, female AD rats showed a significantly lower frequency of entering the open arms of the EPM ($p = 0.0230$) compared to age-matched WT controls (Fig. 1D). Figures 1E and 1F show representative paths of male and female AD and WT rats in the EPM at 9 and 12 months of age. Taken together, these results suggest that anxiety-like behaviors are present in this AD rat model from at least 9 months of age, and that there may be sex-specific differences in the onset of this behavior.

Morris Water Maze

The Morris Water Maze (MWM) was used to assess spatial learning and long-term memory. At 9 and 12 months of age, both male and female AD rats had a significantly greater escape latency ($p < 0.0001$ for all groups) compared to WT rats of the same age and sex (Fig. 2A and 2B). Probe trials of the MWM where the platform was removed from the maze were conducted after 5 days of standard testing. During these probe trials, male AD rats spent significantly less time in the northwestern quadrant (where the platform was previously located) at both 9 months ($p = 0.0001$) and 12 months ($p = 0.0013$) of age compared to age and sex matched WT controls. Female AD rats spent significantly less time in the northwestern quadrant at 9 months of age ($p = 0.0126$) compared to age and sex matched WT rats, but surprisingly no significant difference in the amount of time spent in the northwestern quadrant was detected between 12-month-old females ($p = 0.1089$) and age and sex matched WT rats (Fig. 2C and 2D).

Heat map analysis of 12-month-old Female AD rat and WT rat probe trials revealed that while both groups spent the most time in the northwestern quadrant, the WT group spent more time in a concentrated area while the AD group spent more time roaming the quadrant (Fig. 2E). Taken together, these results indicate that spatial learning and long-term memory deficits can be observed as early as 9 months in this model of AD, and that some sex-specific differences may exist.

Dual Object Recognition Test

The Dual Object Recognition Test was used to assess cognition. At 9 months of age no significant difference was detected in the inspection frequency of a novel object between male AD rats and age matched WTs or female AD rats and age matched WTs. At 12 months of age, both male AD rats ($p = 0.0142$) and female AD rats ($p = 0.0114$) showed significantly lower frequencies of novel object inspection compared to age and sex matched WTs. (Fig. 2F).

Peripheral Metabolic Changes

Quantitative Magnetic Resonance

Quantitative magnetic resonance (QMR) was used to assess body composition and changes in the distribution of body mass. No significant differences in body weight or body fat percentage were detected in 9- or 12-month-old male AD rats compared to age and sex matched WTs (Fig. 3E and 3F). Interestingly, female AD rats had a significantly greater body weight at both 9 ($p = 0.0402$) and 12 ($p = 0.0045$) months of age (Fig. 3A) as well as significantly greater body fat percentages at 9 ($p = 0.0499$) and 12 (0.0029) months of age compared to age and sex matched WT rats (Fig. 3C and 3D). These results strongly suggest that sex specific differences in body composition exist in this model of AD.

Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance testing (IP-GTT) was carried out at 9 and 12 months of age to assess glucose homeostasis. No significant differences in blood glucose were detected between 9-month-old male AD rats or age and sex matched WT rats (Fig. 4A). 9-month-old female AD rats displayed significantly elevated blood glucose levels at 5, 30, and 90 minutes after glucose administration compared to age and sex matched WT rats (Fig. 4B) as well as significantly greater area under the curve (AUC) compared to age and sex matched WT rats ($p < 0.0001$) (Fig. 4C). At 12 months of age, female AD rats displayed greater basal blood glucose levels as well as elevated blood glucose at 5, 15, and 30 minutes after glucose administration compared to age and sex matched WT rats (Fig. 4D) as well as significantly greater AUC compared to age and sex matched WT rats ($p < 0.0001$). Curiously, 12-month-old male AD rats displayed significantly decreased blood glucose at 15, 30, 45, and 60 minutes after glucose administration compared to age and sex matched WT rats (Fig. 4F) as well as significantly reduced AUC compared to age and sex matched WT rats ($p < 0.0001$) (Fig. 4G). These results strongly indicate that sex specific differences in glucose tolerance exist in this model of AD.

Intraperitoneal Insulin Tolerance Test

Intraperitoneal insulin tolerance testing (IP-GTT) was carried out at 9 and 12 months of age to assess insulin sensitivity. No significant differences in blood glucose were detected between 9-month-old male AD rats compared to age and sex matched WT rats after insulin administration (Fig. 5A). 9-month-old female AD rats displayed significantly greater blood glucose concentrations 15, 30, and 45 minutes after insulin administration compared to age and sex matched WT rats (Fig. 5B) as well as significantly increased AUC compared to age and sex matched WT rats ($p < 0.01$) (Fig. 5C). No significant differences were detected in the blood glucose concentration of male AD rats compared to age and sex matched WT rats (Fig. 5D), however 12-month-old male AD rats had significantly elevated AUC ($p < 0.01$) compared to age and sex matched WT rats (Fig. 5E). 12-month-old female AD rats displayed significantly greater blood glucose concentrations compared to age and sex matched WT rats at 30, 45, and 60 minutes after insulin administration (Fig. 5F) as well as significantly greater AUC ($p < 0.001$) compared to age and sex matched WT rats. These results strongly suggest that sex specific differences in insulin sensitivity exist in this model.

Discussion

Alzheimer's disease has been hypothesized to be associated with metabolic dysregulation both peripheral and cerebral. Prevalence of Insulin resistance and type 2 diabetes increases with age and aging may partially drive these observed pathologies. Insulin resistance in the brain may lead to dementia and neurodegenerative disorders. Peripheral metabolic dysregulation in glucose and insulin metabolism can originate from events taking place in certain brain regions. This signal may be bidirectional with even peripheral metabolic dysregulation hampering brain physiology. Here we investigate for

the first time ever in an Alzheimer's disease rat model whether AD pathology in the brain could drive peripheral metabolic dysregulation and evaluate in a coordinated manner whether this may occur before or after the onset of cognitive deficits or any other symptoms of Alzheimer's disease. We found disruptions in glucose homeostasis and insulin sensitivity at 9 months of age where behavioral symptoms of AD were also observed. At 12 months of age as expected, the metabolic changes were enhanced along with behavioral changes.

In our analysis of anxiety-like behavior, clear differences emerged between the AD male and female rats and their WT counterparts. At both 9 and 12 months of age, AD male and female rats spent significantly less time in the open arms of the EPM (Fig. 1A and 1C). This suggests that anxiety-like behaviors in TgF344 rats is onset within 9 months of age, and is consistent with the findings of Pentkowski et al. (2018), the only other study to investigate anxiety-like behaviors in this model to date. We also show that 9-month-old male AD rats as well as 12-month-old male and female AD rats entered the open arms of the EPM significantly less frequently compared to WT counterparts (Fig. 1B and 1D). This contrasts with previous work done in this model, which found no difference in how frequently AD or WT rats entered the open arms of the EPM (Pentkowsk et al., 2018) however this study employed rats aged between 4 and 7 months. We believe this age dependent change in exploration in the EPM exemplifies apathy, a frequently observed behavior change in AD patients which represents a dysfunction in executive cognition (Landes et al., 2001). Increased apathetic behavior prompted the AD rats to remain in the closed arms of the EPM, and the differences in the onset of this behavior between male and female AD rats suggests that there may be sex specific differences in its manifestation. Further research is needed to elucidate the reason for this difference.

The MWM and Dual Object Recognition (DOR) tests revealed that cognitive decline occurs in the TgF344 model of AD at a much younger age than previously thought. Our study shows that at 9 and 12 months of age, male and female AD rats took longer to find the platform compared to WT counterparts (Fig. 2A and 2B). In the MWM probe trial, where the platform was removed from the maze, 9-month-old male and female AD rats as well as 12-month-old male AD rats spent less time in the quadrant of the MWM where the platform was previously located. Interestingly, 12-month-old females AD and WT rats spent similar amounts of time in the correct quadrant, and subsequent heatmap analysis revealed that both spent most of their time in the MWM in this quadrant (Fig. 2C-2E). The DOR test, in contrast to the MWM, revealed cognitive deficits in AD rats only after 12 months of age, where AD male and female rats showed less interest in a novel object than WT counterparts (Fig. 2F). Taken together, the MWM and DOR testing indicate that impaired cognition is present by 12 months of age in this model of AD. When the TgF344-AD rat model was first established, DOR testing showed that by 24 months there was marked cognitive impairments in AD rats and Barnes Maze testing revealed cognitive impairment was present at 15 months of age in AD rats (Cohen et al.,

2013). Further, MWM testing has revealed cognitive impairments in AD rats at 10-11 months of age (Berkowitz et al., 2018). Our study supports these findings and suggests that cognitive impairments may be present at 9 months of age. Interestingly, deficits in working memory have been shown in TgF344-AD rats as early as 2 months of age when delayed nonmatch-to-sample tasks were employed (Munoz-Moreno, Tudela, Lopez-Gil, & Soria, 2018). Additional investigation is necessary to determine a specific onset of cognitive decline in this AD model.

While measuring body weights prior to the insulin and glucose tolerance testing, we noticed a trend wherein the AD rats typically weighed more than their WT counterparts. Clinical studies have found that patients with dementia sometimes do experience weight gain compared to control patients (Mamhidir, Karlsson, Norberg, & Mona, 2007). Additionally, researchers have also seen that with increasing severity of dementia-like symptoms, there is an increase seen in patients' appetites and the increased cravings for sugary foods may be due to a variety of factors including cognitive dysfunction, decline of daily activities, and neurological symptoms (Kai et al., 2015).

Thus, to investigate the differences in body weight, we conducted quantitative magnetic resonance scans of the rats to analyze body composition. No differences were seen in body weight between WT and AD male rats at 9 and 12 months of age. However, for both 9- and 12-month-old females, we saw that the AD females weighed significantly more than their WT counterparts (Fig. 3A). Because of differences in initial body weights, we used bodyfat percentages to compare distribution of mass in the rats. For female rats it was observed that at both 9 (Fig. 3C) and 12 (Fig. 3D) months of age, the AD rats had significantly higher body fat percentages compared to their WT counterparts. We used bodyfat percentage analyses because it accounted for the differing body weights the rats had. Both 9- and 12-month-old males showed no differences in bodyfat percentages (Fig. 3E and 3F).

It is intriguing to note that our study is the first to analyze differences in body composition of the TgF344-AD rat model. Studies have previously characterized the body composition of other preclinical AD models such as the 5xFAD mouse Tg2576 models and they suggested a trend that is opposite to what we see. Specifically, the Tg2576 mice displayed lower body weights than their WT counterparts at 3 months of age regardless of sex (Ishii et al., 2014). In the 5xFAD mice, the researchers found that male transgenic mice had a lowered body weight than their WT counterparts at 6 and 9 weeks of age but no such body weight differences were reported in female mice (Brandscheid et al., 2017). Thus, both mouse models show that the AD mice are lighter than the WT counterparts. While our results recapitulate the sex differences observed in the Brandscheid study, we see differences in female mice in terms of body weight and not male mice. Our rat model's data conflicts with these mice models in the findings

regarding body composition. However, it does suggest there are differences in energy consumption resulting from the AD phenotype.

In our own studies, we saw that though AD males at 9 months of age show no differences in glucose metabolism (Fig. 4A), the AD females at 9 months of age have a much slower response to the introduction of exogenous glucose than their WT counterparts do. This inability to metabolize glucose as quickly is indicated by the AD females' elevated blood sugar levels post injection (Fig. 4B and 4C). At 12 months of age, the AD females' inability to properly metabolize glucose stays consistent (Fig. 4D and 4E). However, at 12 months of age, the AD males show a hyperactive insulin response to the exogenous glucose, resulting in diminished blood sugar levels in response to the introduction of glucose into the body (Fig. 4F and 4G). This indicates that the male AD rats show an increased tolerance to glucose compared to their WT counterparts, suggesting an overactive insulin response. Further studies need to be done, specifically regarding serum levels of insulin as they may indicate hyperinsulinemia which may lead to later insulin resistance development as the AD worsens.

Prior studies analyzing peripheral metabolism in preclinical models of Alzheimer's disease have only been done in mice. In 3xTg-AD mice, researchers found that 6-month old females showed decreased glucose tolerance while the males did not show any signs of metabolic impairments (Gimenez-Llort et al., 2010). Similarly, we found that at 9 months of age, only females had impairments in glucose metabolism while the males did not show changes in glucose metabolism until 12 months of age. However, another research study found that in the same 3xTg-AD mice, there were no metabolic differences between AD and WT mice at 6 months of age. Rather, differences only appeared at 10 months of age as the female AD mice of this age showed decreased glucose tolerance compared to their WT counterparts (Vandal et al., 2015). Neither study found significant differences in the 3xTg-AD mice in terms of insulin sensitivity. Inconsistencies amongst teams of researchers highlighting the onset of peripheral metabolic impairments are seen in other preclinical AD mice models as well. A team of researchers found that APP/PS1 male mice showed decreased glucose tolerance at ages as early as ten weeks of age as well as decreased sensitivity to insulin (Zhang et al., 2012). Using the same mice models, though others found that differences in glucose tolerance and insulin sensitivity between the AD mice and the WT mice were not present at 3 months; rather they only existed at 6 months of age for male APP/PS1 mice (Pedros et al., 2014). Our study provides evidence that there is a pre-diabetic metabolic phenotype present in the TgF344-AD rat model that onsets at the same time as the behavioral AD symptoms in females, but after behavioral AD symptoms in males. Additionally, for female rats, these differences impact body composition in terms of body fat percentages. However, male TgF344-AD rats do not recapitulate these results. The sex differences in terms of peripheral metabolic impairments are profound and implicate that estrogen plays a role in regulating metabolism in the TgF344-AD rats. Despite the abundance of

research suggesting estrogen is a neuroprotective hormone (Raghava, Das, & Ray, 2017; Dubal, 2002; Zarate, Stevnsner, & Gredilla, 2017), Further research needs to be done in order to better understand why male rats seem to experience less metabolic dysregulation compared to their WT counterparts.

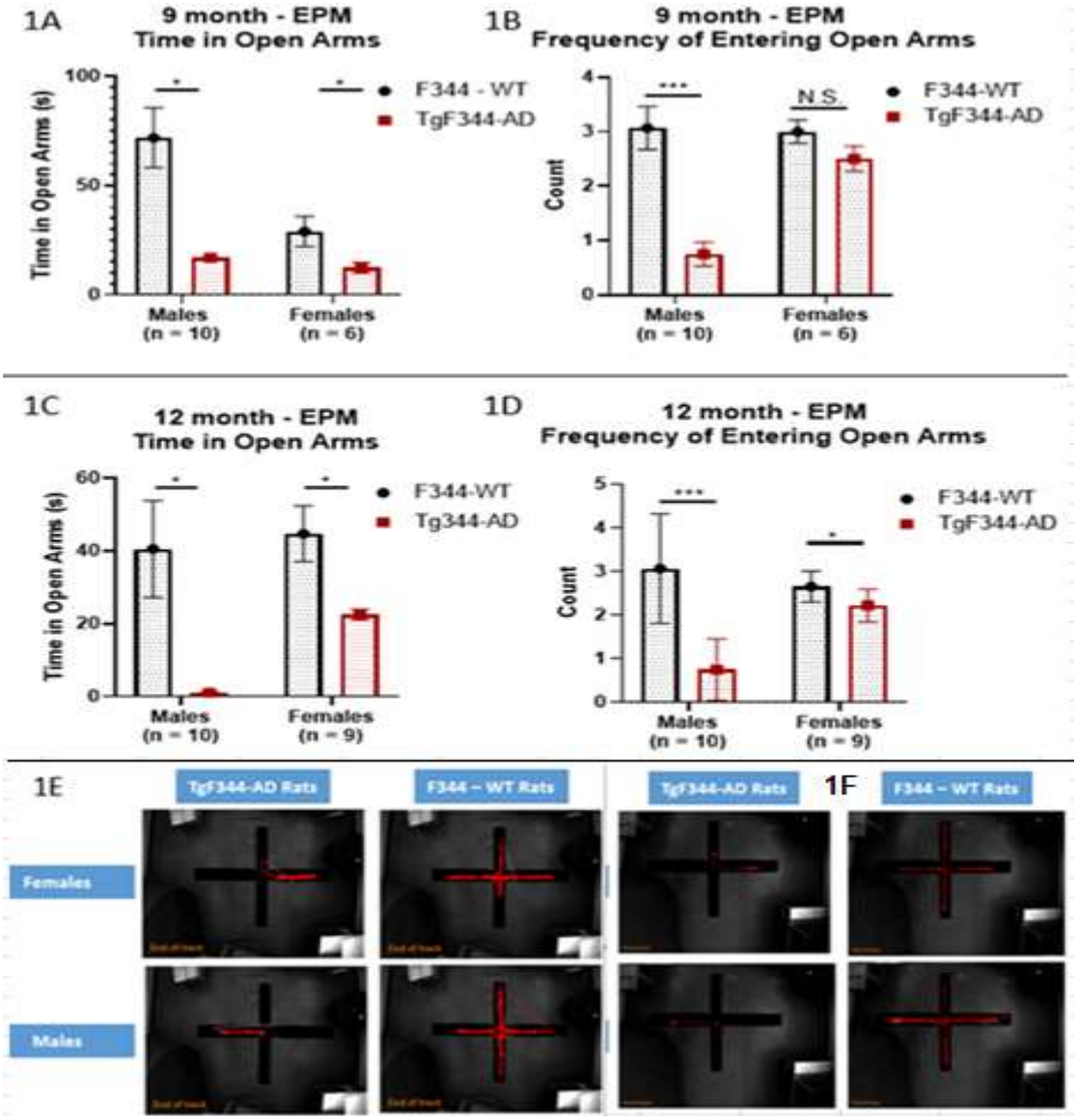
REFERENCES

1. 2020 Alzheimer's disease facts and figures. (2020). 2020 Alzheimer's disease facts and figures. *Alzheimer's & Dementia*, *16*(3), 391–460. https://doi.org/10.1002/alz.12068
2. Kumar A, Sidhu J, Goyal A, et al. Alzheimer Disease. [Updated 2020 Nov 18]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK499922/ (find reference)
3. Kocahan, S., & Doğan, Z.. (2017). Mechanisms of Alzheimer's Disease Pathogenesis and Prevention: The Brain, Neural Pathology, N-methyl-D-aspartate Receptors, Tau Protein and Other Risk Factors. Clinical Psychopharmacology and Neuroscience, 15(1), 1–8. https://doi.org/10.9758/cpn.2017.15.1.1
4. Mehta, D., Jackson, R., Paul, G., Shi, J., & Sabbagh, M.. (2017). Why do trials for Alzheimer's disease drugs keep failing? A discontinued drug perspective for 2010-2015. Expert Opinion on Investigational Drugs, 26(6), 735–739. https://doi.org/10.1080/13543784.2017.1323868
5. Kumar, A., Singh, A., & Ekavali. (2015). A review on Alzheimer's disease pathophysiology and its management: an update. Pharmacological Reports, 67(2), 195–203. doi:10.1016/j.pharep.2014.09.004
6. Kang, S., Lee, Y.-H., & Lee, J. E.. (2017). Metabolism-Centric Overview of the Pathogenesis of Alzheimer's Disease. Yonsei Medical Journal, 58(3), 479. https://doi.org/10.3349/ymj.2017.58.3.479
7. Biessels, G. J., Staekenborg, S., Brunner, E., Brayne, C., & Scheltens, P.. (2006). Risk of dementia in diabetes mellitus: a systematic review. The Lancet Neurology, 5(1), 64–74. https://doi.org/10.1016/s1474-4422(05)70284-2
8. De La Monte, S. M., & Wands, J. R.. (2008). Alzheimer's Disease is Type 3 Diabetes—Evidence Reviewed. Journal of Diabetes Science and Technology, 2(6), 1101–1113. https://doi.org/10.1177/193229680800200619
9. Sperling, R., Mormino, E., & Johnson, K.. (2014). The Evolution of Preclinical Alzheimer's Disease: Implications for Prevention Trials. Neuron, 84(3), 608–622. https://doi.org/10.1016/j.neuron.2014.10.038
10. Cohen, R. M., Rezai-Zadeh, K., Weitz, T. M., Rentsendorj, A., Gate, D., Spivak, I., Bholat, Y., Vasilevko, V., Glabe, C. G., Breunig, J. J., Rakic, P., Davtyan, H., Agadjanyan, M. G., Kepe, V., Barrio, J. R., Bannykh, S., Szekely, C. A., Pechnick, R. N., & Town, T.. (2013). A Transgenic Alzheimer Rat with Plaques, Tau Pathology, Behavioral Impairment, Oligomeric A , and Frank Neuronal

Loss. The Journal of Neuroscience, 33(15), 6245–6256. https://doi.org/10.1523/jneurosci.3672-12.2013

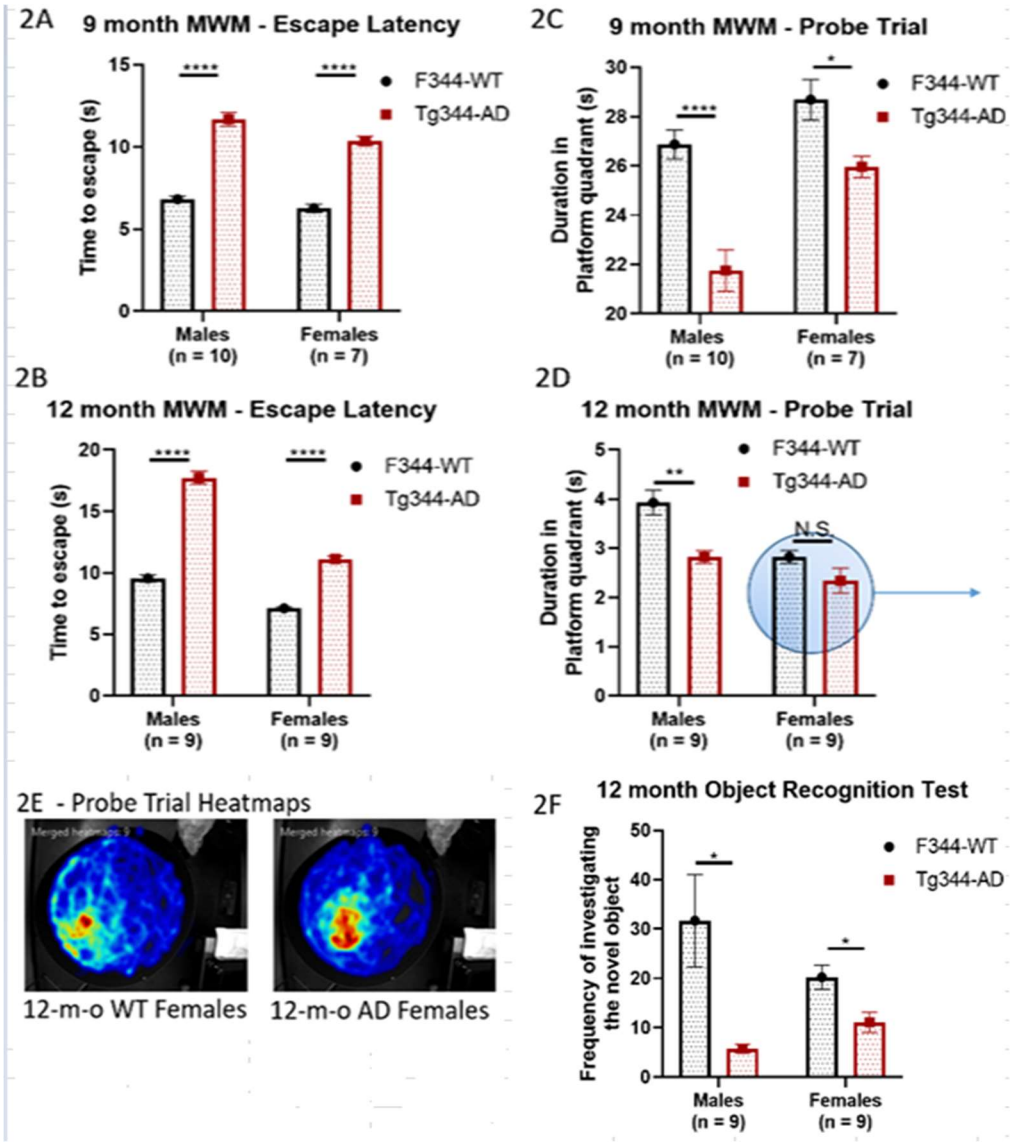
11. Do Carmo, S., & Cuello, A.. (2013). Modeling Alzheimer's disease in transgenic rats. Molecular Neurodegeneration, 8(1), 37. https://doi.org/10.1186/1750-1326-8-37
12. Berkowitz, L. E., Harvey, R. E., Drake, E., Thompson, S. M., & Clark, B. J.. (2018). Progressive impairment of directional and spatially precise trajectories by TgF344-Alzheimer's disease rats in the Morris Water Task. Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-34368-w
13. Muñoz-Moreno, E., Tudela, R., López-Gil, X., & Soria, G.. (2018). Early brain connectivity alterations and cognitive impairment in a rat model of Alzheimer's disease. Alzheimer's Research & Therapy, 10(1). https://doi.org/10.1186/s13195-018-0346-2
14. Macklin, L., Griffith, C. M., Cai, Y., Rose, G. M., Yan, X.-X., & Patrylo, P. R.. (2017). Glucose tolerance and insulin sensitivity are impaired in APP/PS1 transgenic mice prior to amyloid plaque pathogenesis and cognitive decline. Experimental Gerontology, 88, 9–18. https://doi.org/10.1016/j.exger.2016.12.019

Figure 14. Performance on the Elevated Plus Maze in 9 and 12 month rats



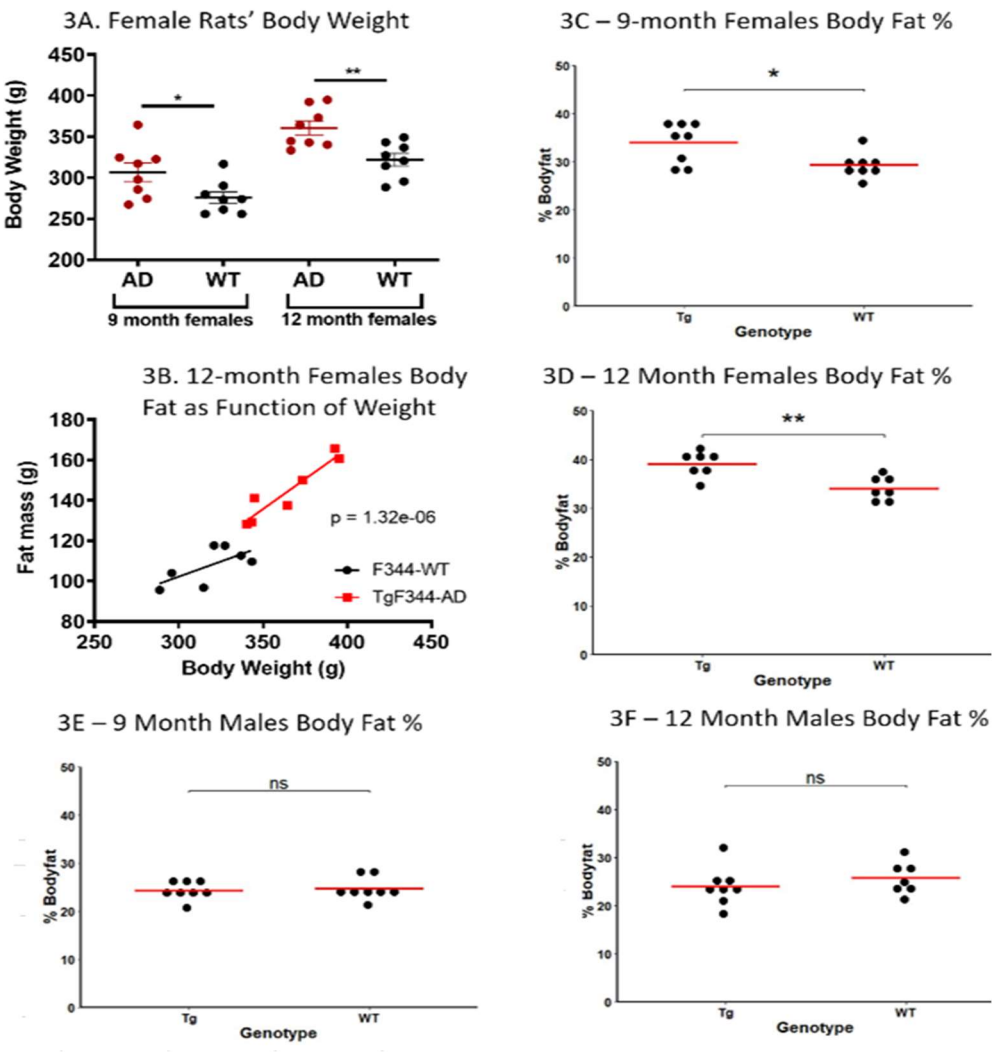
In figures 1A-1D, data is presented as mean ± SEM. Figures 1E and 1F show representative paths for 9- and 12-month-old rats, respectively. Statistical significance was determined by unpaired Student's t-tests where * indicates that $p < 0.05$.

Figure 15. Performance on Morris Water Maze for 9 and 12 month old rats



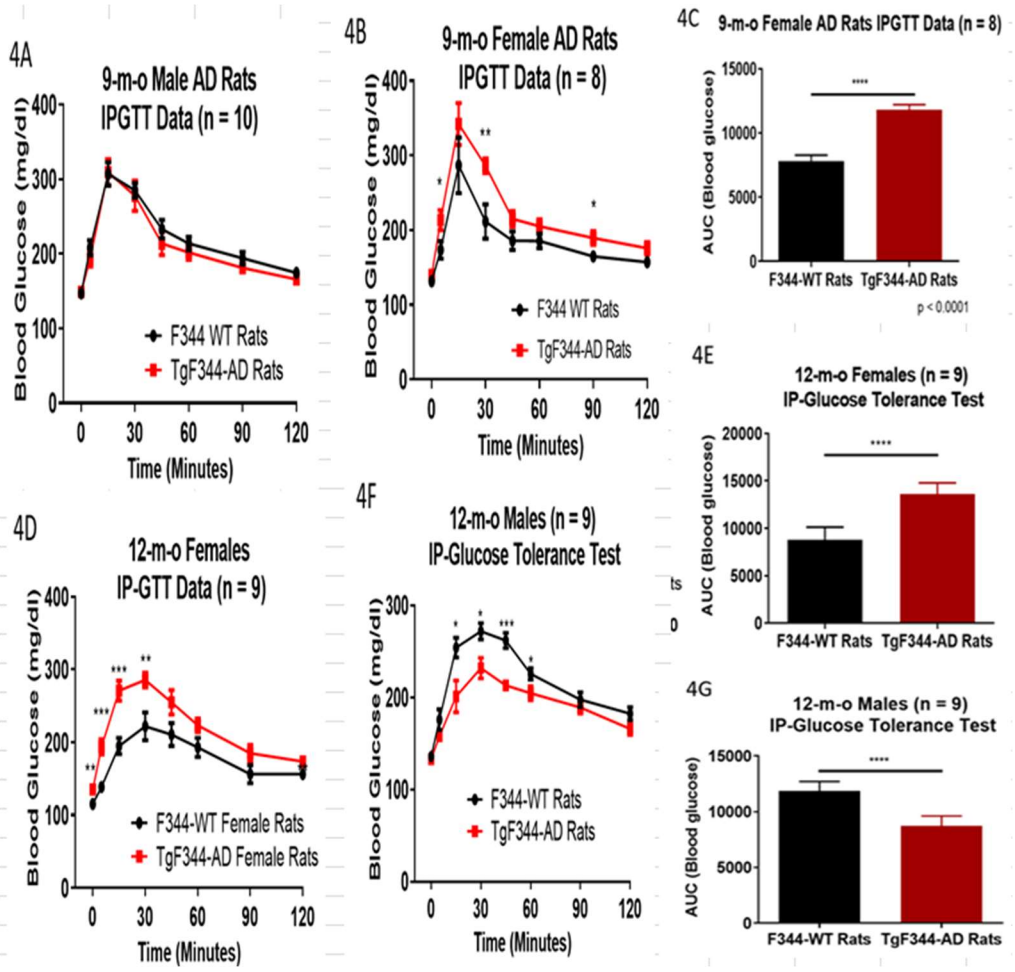
Performance on Morris Water Maze for 9-month-old rats and 12-month-old rats in terms of how long it took to find the platform (2A/2C) and the analysis of roaming behavior in the probe trial (2B/2D). 2E shows average path for 12-month-old WT and AD females. 2F shows results of object recognition test for 12-month-old rats. All data presented as mean ± SEM.

Figure 16. Body composition results from 9 and 12 month old rats



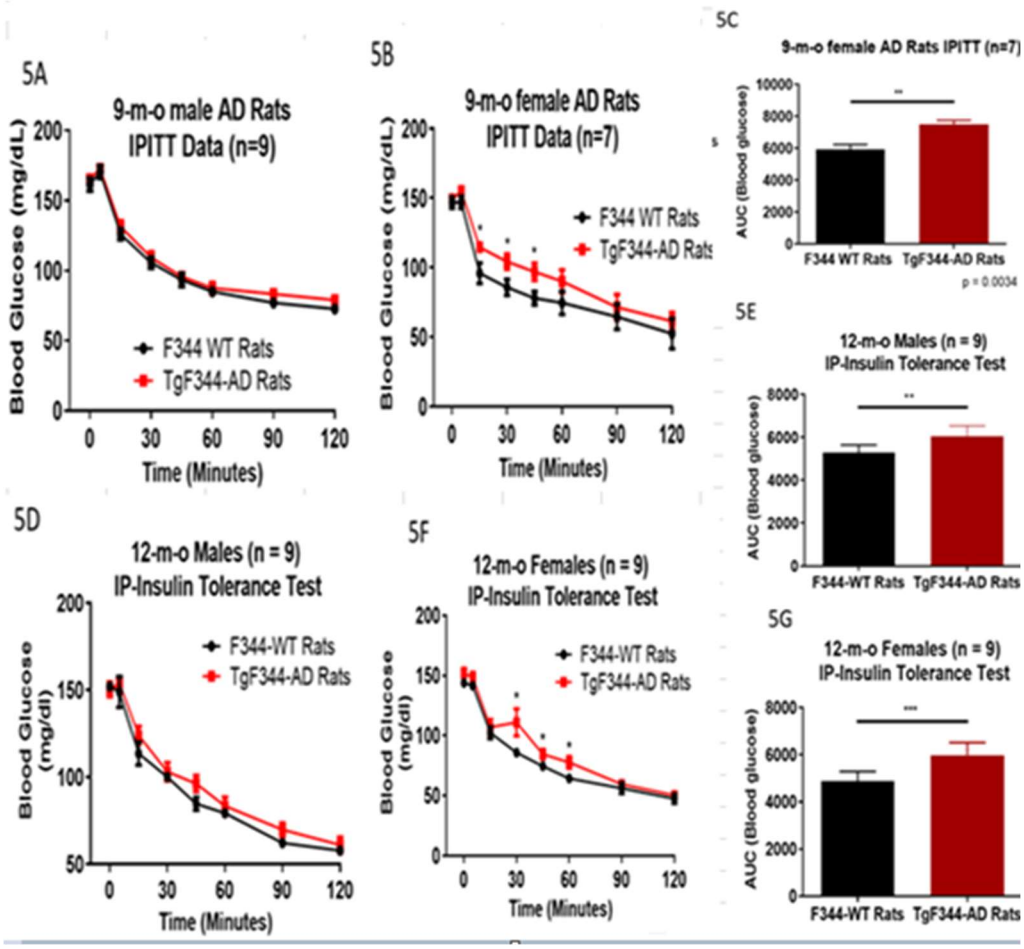
Body composition results from 9 month and 12 month old experimental animals. Data in Fig 3A presented as mean ± SEM and represents body weight for female rats. 3B shows the body weight of 12 month old female rats as a function of body weight. Finally, Figures 3C, 3D, 3E and 3F show body fat percentages for 9-month old females, 12 month old females, 9 month old males and 12 month old males respectively. The bars represent means in 3C-3F.

Figure 17. GTT results of 9 and 12 month old rats



GTT results are shown here. 4A and 4B present blood glucose levels of 9-month-old male and 9-month-old female rats throughout 2 hours post injection respectively. 4C shows AUC analyses for 9-month-old females. 4D shows the blood sugar levels of 12-month-old females 2 hours post injection while 4E shows the AUC analysis for this data. Finally, 4F presents the blood sugar levels of 12-month-old males 2 hours post injection while 4G shows the AUC analysis for this data. Each bar ± SEM. Statistical analyses were performed via unpaired Student's t test with Welch's correction. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$

Figure 18. ITT results of 9 and 12 month old rats



ITT results are shown here. 5A and 5B present blood glucose levels of 9-month-old male and 9-month-old female rats throughout 2 hours post injection, respectively. 5C shows AUC analyses for 9-month-old females. 5D shows the blood sugar levels of 12-month-old males 2 hours post injection while 5E shows the AUC analysis for this data. Finally, 5F presents the blood sugar levels of 12-month-old females 2 hours post injection while 5G shows the AUC analysis for this data. Each bar ± SEM. Statistical analyses were performed via unpaired Student's t test with Welch's correction. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$

GENERAL REFERENCE

1. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The Hallmarks of Aging. Cell 153, 1194–1217 (2013).
2. De Grey, A. D. N. J. A Divide-and-Conquer Assault on Aging: Mainstream at Last. Rejuvenation Research 16, 257–258 (2013).
3. Nadon, N. L., Strong, R., Miller, R. A. & Harrison, D. E. NIA Interventions Testing Program: Investigating Putative Aging Intervention Agents in a Genetically Heterogeneous Mouse Model. EBioMedicine 21, 3–4 (2017).
4. Austad, S. N. The Geroscience Hypothesis: Is It Possible to Change the Rate of Aging? in Advances in Geroscience (eds. Sierra, F. & Kohanski, R.) 1–36 (Springer International Publishing, 2016). doi:10.1007/978-3-319-23246-1_1.
5. Kaeberlein, M. Translational geroscience: A new paradigm for 21st century medicine. Translational Medicine of Aging 1, 1–4 (2017).
6. Hippius, H. & Neundörfer, G. The discovery of Alzheimer's disease. Clinical research 5, 8 (2003).
7. Alzheimer's Association. 2019 Alzheimer's disease facts and figures. Alzheimer's & Dementia 15, 321–387 (2019).
8. Mehta, D., Jackson, R., Paul, G., Shi, J. & Sabbagh, M. Why do trials for Alzheimer's disease drugs keep failing? A discontinued drug perspective for 2010-2015. Expert Opinion on Investigational Drugs 26, 735–739 (2017).
9. Duara, R. et al. A comparison of familial and sporadic Alzheimer's disease. Neurology 43, 1377–1377 (1993).
10. Ricciarelli, R. & Fedele, E. The Amyloid Cascade Hypothesis in Alzheimer's Disease: It's Time to Change Our Mind. CN 15, (2017).
11. Oxford, A. E., Stewart, E. S. & Rohn, T. T. Clinical Trials in Alzheimer's Disease: A Hurdle in the Path of Remedy. International Journal of Alzheimer's Disease 2020, 1–13 (2020).
12. Razay, G., Vreugdenhil, A. & Wilcock, G. The Metabolic Syndrome and Alzheimer Disease. Arch Neurol 64, 93 (2007).
13. Leibson, C. L. et al. Risk of Dementia among Persons with Diabetes Mellitus: A Population-based Cohort Study. American Journal of Epidemiology 145, 301–308 (1997).
14. Ronnemaa, E. et al. Impaired insulin secretion increases the risk of Alzheimer disease. Neurology 71, 1065–1071 (2008).

15. Feart, C., Samieri, C. & Barberger-Gateau, P. Mediterranean diet and cognitive health: an update of available knowledge. Current Opinion in Clinical Nutrition and Metabolic Care 18, 51–62 (2015).
16. Knight, E. M., Martins, I. V. A., Gümüsgöz, S., Allan, S. M. & Lawrence, C. B. High-fat diet-induced memory impairment in triple-transgenic Alzheimer's disease (3xTgAD) mice is independent of changes in amyloid and tau pathology. Neurobiology of Aging 35, 1821–1832 (2014).
17. Inelmen, E. M., Sergi, G., Coin, A., Girardi, A. & Manzato, E. An open-ended question: Alzheimer's disease and involuntary weight loss: which comes first? Aging Clin Exp Res 22, 192–197 (2010).
18. Reuter, J. A., Spacek, D. V. & Snyder, M. P. High-Throughput Sequencing Technologies. Molecular Cell 58, 586–597 (2015).
19. Parashar, A. & Udayabanu, M. Gut microbiota: Implications in Parkinson's disease. Parkinsonism & Related Disorders 38, 1–7 (2017).
20. Clemente, J. C., Ursell, L. K., Parfrey, L. W. & Knight, R. The Impact of the Gut Microbiota on Human Health: An Integrative View. Cell 148, 1258–1270 (2012).
21. Cox, L. M. & Weiner, H. L. Microbiota Signaling Pathways that Influence Neurologic Disease. Neurotherapeutics 15, 135–145 (2018).
22. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. 10.
23. Sampson, T. R. et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell 167, 1469-1480.e12 (2016).
24. Vogt, N. M. et al. Gut microbiome alterations in Alzheimer's disease. Sci Rep 7, 13537 (2017).
25. Zhuang, Z.-Q. et al. Gut Microbiota is Altered in Patients with Alzheimer's Disease. JAD 63, 1337–1346 (2018).
26. Cohen, R. M. et al. A Transgenic Alzheimer Rat with Plaques, Tau Pathology, Behavioral Impairment, Oligomeric A , and Frank Neuronal Loss. Journal of Neuroscience 33, 6245–6256 (2013).

APPENDIX

IACUC Approval Form

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 06-Apr-2020

TO: Sun, Liou Y

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 06-Apr-2020.

Protocol PI:	Sun, Liou Y
Title:	The Effects of Hormonal, Dietary and Pharmacological Signals on Mammalian Aging
Sponsor:	National Institute on Aging/NIH/DHHS
Animal Project Number (APN):	IACUC-20292

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 05-Apr-2023.

Institutional Animal Care and Use Committee (IACUC)

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