Published in final edited form as:

Neurotox Res. 2019 February ; 35(2): 291–303. doi:10.1007/s12640-018-9954-7.

Development of a human *APOE* knock-in mouse model for study of cognitive function after cancer chemotherapy

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Abstract

Cancer-related cognitive impairment in breast cancer patients exposed to multi-agent chemotherapy regimens is associated with the Apolipoprotein E4 (*APOE4*) allele. However, it is difficult to determine the effects of specific agents on cognitive impairment in human studies. We describe the development of a human *APOE* knock-in congenic C57BL/6J mouse model to study cancer-related cognitive impairment. Female *APOE3* and *APOE4* homozygous mice were either left untreated or treated with the most commonly used breast cancer therapeutic agent, doxorubicin. *APOE3* and *APOE4* mice had similar behaviors in exploratory and anxiety assays, which were affected transiently by doxorubicin treatment. Spatial learning and memory were measured in a Barnes Maze: after four days of training, control *APOE3* and *APOE4* mice were able to escape with similar latencies. In contrast, doxorubicin-treated *APOE4* mice had markedly impaired learning compared to doxorubicin-treated *APOE3* mice at all time points. Voxel based morphometry of magnetic resonance images revealed that doxorubicin treatment caused significant changes in the cortex and hippocampus of in both *APOE3* and *APOE4* mouse brains, but the differences were significantly greater in the *APOE4* brains. The results indicate that doxorubicin-exposed *APOE4* mice recapitulate key aspects of human cancer-related cognitive impairment. These data support the usefulness of this novel pre-clinical model for future elucidation of the genetic and molecular interactions of *APOE* genotype with chemotherapy; this model can also allow extension to prospective studies of older mice to study these interactions in the context of aging.

Keywords

cancer-related cognitive decline; *APOE*; chemotherapy; preclinical model

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Conflicts of interest

The authors declare that that they have no conflicts of interest.

Statement on the welfare of animals

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of Georgetown University, where these studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Introduction

Cancer and neurodegenerative diseases account for the majority of morbidity, mortality, and healthcare costs in the US among older individuals (Dewey and Saz 2001; Mariotto et al. 2011). There are presently approximately 15.5 million cancer survivors living in the US (Bluethmann et al. 2016; Miller et al. 2016; Rowland and Bellizzi 2014), and two-thirds of these survivors are aged 65 and older (Mariotto et al. 2011). The process of aging places these older cancer survivors at risk of neurodegenerative disease (Mandelblatt et al. 2014b) and its associated cognitive impact.

Human and animal studies suggest that cancer chemotherapeutic agents can lead to alterations in biomarkers of aging (Sanoff et al. 2014), cause cognitive decline (Kesler and Blayney 2016; Sanoff et al. 2014), and result in brain atrophy that mimics degenerative age-related changes (Kesler and Blayney 2016; Mandelblatt et al. 2013; Mandelblatt et al. 2014b; Nudelman et al. 2016). Hence, older cancer survivors are likely to be particularly vulnerable to experiencing cognitive decline as a result of age and cancer and its treatments.

Recent data from our group and that of others have shown that having an *E4* allele of the *APOE* (Apolipoprotein E) gene is a risk factor for chemotherapy-related cognitive decline (Ahles et al. 2014; Ahles et al. 2010; Mandelblatt et al. 2018). This finding is highly relevant given that the *APOE4* allele is present in approximately 70 million Americans (about 25% of the population), affects normal brain function and structure, and is the strongest genetic risk factor for Alzheimer's disease (Raber et al. 2004). The *APOE4* allele also affects brain inflammatory processes and plasticity and repair mechanisms important in aging and neurodegeneration (Heppner et al. 2015; Malik et al. 2015; Namjoshi et al. 2013; Zhong et al. 2016).

Longitudinal studies in older cancer survivor populations are useful to investigate the complex relationships between genetic factors, cancer and its treatment, and aging with potential molecular and pathophysiological mechanisms of cognitive decline. However, it is unlikely that sufficiently large cohorts of patients can be accrued to define individual drug-specific effects as well as the mechanistic underpinnings of these interactions, given the numbers and combinations of therapeutic agents (Seigers et al. 2015), the effects of different lifestyle factors, and the long follow-up period needed to define aging effects on cognitive behavior and brain structure and function. In such a situation, preclinical animal models that are built upon human observations can be useful to accelerate discovery (Seigers et al. 2015).

Prior studies of preclinical models of cancer-related cognitive decline have identified adverse effects of doxorubicin on measures of memory (Seigers et al. 2015), hippocampal neurogenesis (Acharya et al. 2015; Christie et al. 2012; Wigmore 2013), hippocampal volumes (Seigers and Fardell 2011; Seigers et al. 2016), oxidative stress (Keeney J 2018), and inflammation (Tangpong et al. 2007; Tangpong et al. 2011). However, only one of these studies included older animals (Dubois et al. 2014), and none have assessed longitudinal changes in female mice, or considered genetic risk factors related to cognitive decline.

We previously described the analysis of several brain and behavioral phenotypes of a human *APOE* knock-in mouse model that supports the normal expression and regulation of the human *APOE3* and *APOE4* alleles (Sullivan et al. 2004). The *APOE4* mice have significant deficits in neuronal structure and cognition compared to *APOE3* mice, as well as higher levels of pro-inflammatory cytokines in response to various brain insults (DiBattista et al. 2016; Rodriguez et al. 2013; Rodriguez et al. 2014; Tai et al. 2015; Zhao et al. 2014; Zhou et al. 2015; Zhu et al. 2012). Thus, this mouse model is an excellent preclinical option for studying the phenotypes of normal individuals possessing the *APOE3* and *APOE4* alleles.

In this study, we describe the development of a biologically and clinically relevant mouse model of cancer-related cognitive decline with the goal of better defining the effects of specific *APOE* alleles in this disorder. We identify *APOE* genotype differences among untreated mice and mice exposed to doxorubicin, focusing on spatial learning behavior and on hippocampal and cortical volumes. These domains are particularly relevant to effects seen in humans experiencing cognitive impairment after chemotherapy. Data from this new mouse model will ultimately help elucidate the molecular mechanisms by which specific cancer treatments interact with *APOE4* in the context of aging to impact cognitive function.

Methods

Ethics Statement

This study was conducted in accordance with ethical standards and relevant national and international guidelines for animal welfare, including the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures and handling of the animals were also performed according to protocols approved by the Georgetown University Medical Center Institutional Animal Care and Use Committee.

Study Design

This preclinical study followed the recent recommendations of the International Cognition and Cancer Task Force for harmonization of preclinical studies (Winocur et al. 2018). We tested for differences in behavior and brain structure by *APOE* genotype in untreated mice and in doxorubicin-treated mice. Doxorubicin is the most commonly used chemotherapeutic agent used in breast cancer and one of the most frequently implicated in cancer-related cognitive decline in human (Kesler and Blayney 2016) and animal studies (Seigers et al. 2016). We compared behaviors in untreated mice at one time point, and in doxorubicin-treated mice at time points relative to a single exposure to doxorubicin: pre-exposure, and one and six weeks after exposure. Mice were euthanized after behavioral testing and whole brains were prepared for imaging.

Animal Population

Human *APOE3* (n=18) and *APOE4* (n=21) knock-in C57BL/6J female mice (Jackson Laboratories, Inc., Bar Harbor, ME) were maintained at Georgetown University. Congenic C57BL/6J mice are the most commonly used in studies of cognition based on their intermediate phenotype in spontaneous behavior and good learning skills (Brooks et al. 2005; Loos et al. 2014; Seigers et al. 2015). As we were testing a chemotherapeutic agent

used for breast cancer, we limited our experiment to females (Bimonte-Nelson et al. 2003). The animals were bred as homozygotes. The *APOE3* mice are equivalent to humans with the *APOE3.3* genotype (64% of the US population), and the *APOE4* mice are equivalent to humans with the *APOE4.4* genotype (2% of the US population) (Ward et al. 2012). The *APOE4* allele is the strongest genetic risk factor for late onset Alzheimer's disease (Raber et al. 2004). Studies with doxorubicin were initiated in mice at approximately 14 weeks of age and completed at 21 weeks of age; control mice were analyzed at 22 to 25 weeks of age.

Staff involved in breeding, genotype verification, tagging, colony maintenance and chemotherapy administration were separate from those involved in the behavioral testing, imaging, and analyses. The latter personnel were blinded to genotype and chemotherapy exposure status. Mice were housed in cages with littermates and were kept on a 12-hour light/dark cycle and had *ad libitum* access to water and rodent chow.

Treatment

Mice were weighed weekly throughout the experiment. Doxorubicin (10mg/kg; Abcam, PLC, Cambridge, UK) was prepared in DMSO under sterile conditions for a single tail vein injection volume of 100 μ L. The control animals were left untreated. The health of each animal, based on weight, general appearance, level of activity, and signs of inflammation due to injection, was checked daily. Animals that experienced tail erythema had antibiotic ointment applied three times per week until the condition improved. The open field and elevated plus tests were performed one week prior to treatment, and one and six-weeks post-treatment.

Measures and Testing Procedures

Weight, general and neurological behaviors, and *ex vivo* histological organ changes were used as measures of chemotherapy toxicity. The outcome measures included behavioral assays and MRI-quantified cerebral morphological alterations.

Behavioral Assays

Open Field Test: An open field measuring 45 cm x 45 cm was used one week prior to treatment and one- and six-weeks post-treatment (or contemporaneously for controls) to measure general behavior, including anxiety, exploration, and locomotion. The field was illuminated evenly at 100 lux by overhead lighting in a quiet room with no external cues. Animals were positioned in the center of the open field and allowed to explore the apparatus for five minutes. Overall locomotor activity (path length) was recorded by an overhead camera and analyzed using the Anymaze behavioral tracking package (Stoelting Co., Wood Dale, IL). The number of animal rears was also recorded to measure general vigilance and exploratory drive.

Elevated Plus Maze: The elevated plus maze was used as a measure of anxiety. The maze, with arms measuring 30 cm in length and 30 cm in height, was placed in a quiet room and illuminated evenly at 100 lux by overhead lighting. The mice were placed in the center of the maze facing an open arm and allowed to explore the maze for five minutes. The duration of time spent in the open arms was recorded by an overhead camcorder and data was

analyzed using Anymaze software suite. Time in exposed arms of the maze indicates lower levels of anxiety.

Barnes Maze: The Barnes maze (San Diego Instruments Inc., San Diego, CA) was used to measure spatial learning and memory. Given the known strong practice effects, the Barnes maze was used once, during the fifth week after treatment, or during the contemporaneous age for controls. To minimize variability, all testing was done by the same investigator at the same time of day, and on the same apparatus.

The maze was placed in the center of a room with four extra-maze cues. Using established Barnes maze protocols (Rosenfeld and Ferguson 2014), four lights provided 150 lux of illumination to the maze and an overhead radio played 75 dB of white noise in order to provide aversive stimuli during trials. Out of twenty possible escape holes, one escape was randomly chosen and fitted with a 10 cm x 4 cm x 4 cm escape container. All other escape locations contained shallow decoy escape boxes. The mice were placed in the center of the maze under a darkened canopy for ten seconds prior to each trial. During the habituation phase, the mice were allowed to explore the maze for a single 180-second trial and were gently guided to the escape hole if they failed to locate it before the end of the trial. The mice repeated these trials four times per day over the next four days, with trials spaced 15 minutes apart. The time required to learn the location of the escape hole for each trial (latency, in seconds) was used to measure spatial learning.

To examine whether the mice were using external spatial cues to locate the hole, a blinded observer also categorized the learning strategy of each mouse (random, serial, or direct) on each training day. A direct strategy was defined as one in which the mouse moved directly into the target quadrant, crossed the maze center less than twice, and made three or fewer incorrect nose pokes at a decoy hole (Rosenfeld and Ferguson 2014). The target hole, each decoy hole, and the maze itself were cleaned with a mild soap and water solution between each trial in order to eliminate olfactory cues.

A single probe trial was conducted 72 hours after the above final testing trial, during which the previous escape hole was replaced with a twentieth decoy escape to test spatial memory. This trial was stopped once the animal entered the correct decoy escape location or after 180 seconds. Animal behavior (time to find the former escape hole location) was recorded by an overhead camcorder and analyzed using Anymaze.

Magnetic Resonance Imaging (MRI)—After completion of all behavioral testing, mice were euthanized using a CO₂ chamber and transcardially perfused with 50mL of 10% neutral buffered formalin. For the control animals, six APOE3 and six APOE4 whole brains were harvested for MRI. For the treated groups, the whole brains from approximately 50% of the animals of each genotype (five APOE3 and six APOE4), were extracted and embedded in 3% agar in PBS for *ex vivo* MRI, as described previously (Lee et al. 2018). Three-dimensional *ex vivo* MRI was performed in order to analyze differences in regional brain anatomy using voxel-based morphometry (VBM) (Ashburner and Friston 2000). Briefly, MRI was performed in the Georgetown-Lombardi Preclinical Imaging Research Laboratory in a 7-tesla Bruker spectrometer (Bruker Biospin, Ettlingen, Germany) running

Paravision 5.1 software, with a 32 mm mouse brain volume coil. A 3-D rapid acquisition with relaxation enhancement (RARE) T2-weighted sequence was used to assess anatomical changes with the following: repetition time = 2500 ms, effective echo time = 36 ms, RARE factor = 12, flip angle = 90 degrees, field of view = $17.5 \times 17.5 \times 20 \text{ mm}^3$, matrix size = $190 \times 190 \times 170$, and spatial resolution of $92 \times 92 \times 118 \text{ mm}^3$.

Voxel-based Morphometry (VBM)—For the analysis of the 3-D MRI datasets, the 2dseq raw images were first converted to Bruker Dicom files, exported and converted to NIfTI format files using MRICron software. Images were skull-stripped using MIPAV 7.1.1 software. The images were aligned to the SPMouse mouse brain template via affine transformation with 12 parameters (translations, shears, rotation and scale factors in three dimensions) using FSL (FMRIB Software Library, Oxford, UK). Based on the unified segmentation algorithm, each image was segmented into grey matter (GM), white matter using SPMouse. The warped modulated GM maps were smoothed with a 0.5 mm FWHM Gaussian kernel.

These data were used to test for group-wise differences in the GM maps across the whole brain with SPMouse toolbox by performing a voxel-by-voxel two-sample t-test and f-test. Whole brain analysis was reformed with a significance level of $p < 0.03$, uncorrected for multiple comparisons.

Data Analysis—Bivariate analyses were used, including independent sample t-tests, one-way ANOVA, and Fisher's exact tests to compare the mice by genotype and exposure group with respect to baseline pre-exposure and post-exposure behaviors; a p value of <0.05 was considered statistically significant. Doxorubicin-treated *APOE3* and *APOE4* mice were analyzed behaviorally at a separate time than the untreated controls, so direct statistical comparisons only examined the effects of *APOE* genotype in doxorubicin-treated mice or of *APOE* genotype in control mice groups. In contrast, VBM statistical analyses were conducted both within and between groups, due to the low variability of this assay over time. Analysis of behavioral statistics and figure generation of these data was performed using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA).

Results

The average weight of the mice was 18.5g (SD 1.3g) and did not vary by *APOE* genotype. After a single injection of doxorubicin, all the treated mice maintained their body weight or showed a small weight loss ($<10\%$ of body weight). There was no significant weight difference between untreated *APOE3* or *APOE4* control mice before or after behavioral testing. Four of the treated mice (two *APOE3*, two *APOE4*) displayed local irritation surrounding the tail-vein injection site. There were no treatment-related deaths.

There were no differences in exploration and anxiety by genotype in control mice: *APOE4* mice displayed a similar drive in exploring a novel environment (open field) as their *APOE3* counterparts, based on the total distance travelled within the field (Figure 1A) and rearing events in the same open field (Figure 1C). Likewise, there were no significant differences observed between *APOE* genotypes in a test of rodent anxiety (elevated plus; Figure 1E).

There were no differences in exploration and anxiety by genotype at baseline in the treatment group (Figures 1B, D, F). One week after treatment with doxorubicin, both *APOE3* and *APOE4* mice showed a significantly decreased willingness to explore the open field (both distance traveled and rearing) compared to their pre-exposure behaviors. By six weeks post-treatment, *APOE3* mice returned to their pre-test exploratory levels, whereas *APOE4* mice maintained a significant reduction compared to their pre-treatment behaviors (Figure 1B & 1D, $p < .05$). There were no differences in post-treatment anxiety by genotype as measured by time spent in the open arms of the elevated plus maze (Figure 1F).

Cognitive Behavior Outcomes

Controls—There were significant differences by *APOE* genotypes in spatial learning and memory in control mice. During each training day in the learning phase, *APOE4* mice displayed significantly poorer spatial learning (i.e., the ability to locate the target escape hole in the Barnes maze) when compared to *APOE3* mice ($p < 0.01$). This difference decreased by training day 4, but nonetheless remained significant (*APOE3* mice: 11 seconds; *APOE4* mice: 22 seconds, $p < 0.01$) (Figure 2A). A 72-hour probe trial, designed to measure long-term retention of the target hole location, likewise revealed a significant difference in the ability to locate the target hole between *APOE* genotypes ($p < 0.01$) (Figure 2B). Analysis of the search strategies showed that the proportion of mice using a direct strategy in any given trial, indicating accurate retention and use of the external spatial cues, increased as animals learned the maze from training day 1 to day 4 (Figure 3A). Control *APOE3* mice showed higher use of a direct strategy compared to control *APOE4* mice, although, on the final training day, there was no significant difference between *APOE* genotypes.

Chemotherapy-exposed

Five weeks post-exposure, doxorubicin-treated *APOE4* mice, compared to doxorubicin-treated *APOE3* mice, took a significantly longer time to locate the target hole (latency) on each of the four training days (16 total trials; Figure 2C). By training day 4, the *APOE3* mice located the target hole in an average of 16 seconds, significantly less than the nearly 100 seconds for the *APOE4* mice ($p < 0.001$). Differences between the chemotherapy-treated *APOE3* and *APOE4* mice were observed as early as the first training day; treated *APOE3* mice took less time to locate the target hole compared to treated *APOE4* mice on all but the first trial (Figure 2C, inset). In the 72-hour probe trial, treated *APOE4* mice exhibited significantly greater time than treated *APOE3* mice to locate and enter the escape target hole three days after the last training day, as expected given their poor learning over the training days ($p < 0.01$, Figure 2D). The proportion of doxorubicin-treated *APOE3* mice using a direct strategy to locate the hole was significantly greater than *APOE4* mice on the third and fourth training days ($p < 0.05$, Figure 3B).

Control and doxorubicin-treated *APOE3* mice showed similar patterns of spatial learning from training days 1 to 4 (Table 1). Control and doxorubicin-treated *APOE4* mice showed decreased spatial learning ability in the Barnes maze when compared to the corresponding *APOE3* mice. When expressed as a percentage of total allowed time taken to enter the target hole (180 seconds), both unexposed and doxorubicin-treated *APOE3* treatment groups were able to enter the target hole relatively quickly at the end of the test compared to the

beginning of the test (7% and 9% of the time, respectively). Likewise, unexposed *APOE4* mice were able to locate and enter the target hole within 12% of their total allotted time on training day 4. However, *APOE4* mice exposed to doxorubicin showed significantly longer latencies to locate and enter the correct escape hole; with 55% of the allotted trial time to escape the Barnes maze, even by day four (Table 1).

Brain Structure

In order to investigate morphological cerebral alterations associated with chemotherapy, we performed anatomical MRI of both unexposed and doxorubicin-treated *APOE3* and *APOE4* mice and conducted whole-brain analysis using voxel-based morphometry. Group comparisons of GM volume revealed that, compared to control *APOE3* mice, control *APOE4* mice had several significantly smaller grey matter volumes bilaterally, including dorsal portions of the hippocampus and regions of the striatum (Figure 4A,B). Furthermore, compared to doxorubicin-exposed *APOE3* mice, the doxorubicin-exposed *APOE4* mice had significantly smaller grey matter volumes bilaterally in the dorsal and ventral hippocampus, the striatum, the piriform cortex as well as the lateral geniculate nucleus (Figure 4C,D). Interestingly, these comparisons across *APOE* genotypes showed larger affected hippocampal regions in doxorubicin-treated *APOE4* mice (Figure 4B,D).

These observations led us to directly test the effects of doxorubicin treatment on *APOE3* mice and on *APOE4* mice, by comparing the doxorubicin-exposed brains to the unexposed brains (Figure 5). Based on the VBM analysis of 3-D whole brain image datasets, we found that after doxorubicin treatment, the *APOE3* mice showed significant differences in grey matter bilaterally in the frontal cortex and throughout the hippocampus (Figure 5A,C). Importantly, the *APOE4* mice showed a very similar pattern of grey matter alterations compared to the *APOE3* mice. These alterations for the *APOE4* mice were markedly stronger than for the *APOE3* mice (Figure 5B,D). These observations are consistent with Figure 4D showing differences in hippocampal volumes in the doxorubicin-treated *APOE4* mice compared to the doxorubicin-treated *APOE3* mice.

Discussion

Several prominent genetic factors predispose individuals to different types of neurodegenerative diseases; recent studies have suggested that the same may be true for cognitive decline after cancer chemotherapy (Snyder et al. 2017). Here we report the first study to apply a human *APOE* knock-in rodent model to examine the possible interactions of the *APOE* genotype with chemotherapeutic agents and the effect of this association on cognitive behavior and brain structure. We found that *APOE4* mice treated with doxorubicin, a standard of care breast cancer chemotherapy agent, exhibited an impairment of behaviors and alterations in brain structure compared to their *APOE3* counterparts and untreated control *APOE4* mice. These results suggest that this preclinical model could be further developed into a novel translational *in vivo* platform for defining the mechanisms of chemotherapy-related cognitive impairment using additional chemotherapeutic agents, both as single agents and in combination treatments, recapitulating regimens frequently used in the clinic. In addition, this model provides the experimental foundation for directly assessing

the combined effects of aging and *APOE* status on chemotherapy-induced cognitive impairment.

In past pre-clinical studies, doxorubicin (Salas-Ramirez et al. 2015) and other breast cancer chemotherapy agents (Foley et al. 2008; Yang et al. 2010) caused decrements in cognitive behaviors (Seigers et al. 2015) and adverse effects on brain structure or growth in mice (Acharya et al. 2015; Christie et al. 2012; Seigers and Fardell 2011; Seigers et al. 2016; Wigmore 2013). For example, Seigers and colleagues (2015) found impairment in spatial memory in male C57BL/6J mice two weeks after doxorubicin treatment with a similar dose to that used in our study. Our results are consistent with these past studies, despite differences in the experimental design and genetic background, and extend these results to suggest a gene-treatment interaction in effects on cognition.

The *APOE* allele is the best characterized genetic factor to date associated with an increased risk of cancer-related cognitive decline (Ahles et al. 2014; Ahles et al. 2003; Mandelblatt et al. 2014b). There are three major *APOE* alleles, with *APOE4* being associated with an increased risk and earlier onset of Alzheimer's disease (Liu et al. 2013). *APOE3* is the most common allele, and thus, the *APOE3* knock-in mice represent an accurate population-based control group for our preclinical model. Importantly, neither *APOE3* nor *APOE4* mice develop the brain lesions associated with Alzheimer's disease (amyloid plaques and neurofibrillary tangles), and therefore, they are excellent models of patients without frank neurodegeneration, which would be a confounding factor for studies of behavior and brain atrophy.

Reduced hippocampal volume has been observed in human neuroimaging studies of both cancer-related cognitive decline and Alzheimer's disease (Nudelman et al. 2016; Saykin et al. 2013; Schuff et al. 2009). In this study, we compared the hippocampus of *APOE3* and *APOE4* mice, both in the absence and in the presence of doxorubicin treatment. Unexposed *APOE4* mice had a smaller hippocampus bilaterally compared to unexposed *APOE3* mice, consistent with human imaging data of *APOE4* control individuals (Reiman et al. 1998). After we exposed mice to doxorubicin, we found that the hippocampus and frontal cortex volumes were significantly impacted in size in both *APOE3* and *APOE4*, but the changes were more severe in the *APOE4* mice. These data demonstrate that doxorubicin may have a more significant impact on the hippocampus of individuals with an *APOE4* than those with an *APOE3* allele. The mammalian hippocampus is one of the few areas within the central nervous system where neurogenesis may occur throughout the lifespan of the animal (Bruehl-Jungerman et al. 2007; Spalding et al. 2013). Administration of cytotoxic agents (e.g. doxorubicin) may directly disrupt neurogenesis in the rodent hippocampus and impair performance on measures of spatial learning and memory (Lucassen 2016). Since hippocampal volume decreases with aging, it is possible that differences between *APOE* genotypes may be more pronounced in older animals than those observed in the current study performed on young mice; we are actively investigating this possibility.

Behavioral tests in doxorubicin-treated *APOE4* mice showed impaired spatial learning via the Barnes maze, correlating with the observed significant alterations in grey matter in the hippocampus. Unexposed *APOE4* mice also showed deficits in the Barnes maze compared

to unexposed *APOE3* mice but to a lesser degree (Di Battista et al. 2016; Rodriguez et al. 2013), consistent with our finding of smaller hippocampal volumes in unexposed *APOE4* mice. Other behaviors, such as motor control, reward seeking behavior, and visual acuity, were not measured through the behavioral tests administered. Future behavioral assessments could determine how well each of the VBM findings in our mouse model correlate with the neurological effects of chemotherapeutics. They also suggest that VBM can potentially be applied as an objective imaging biomarker of cancer-related cognitive decline in humans for improved diagnosis, prognosis and treatment. Future research directions include investigating the utility of MRI to detect functional and metabolic alterations in our mouse model of cancer-related cognitive decline, which might enhance the sensitivity of non-invasive imaging in the diagnosis of this disorder.

The human *APOE* knock-in mouse model has contributed to our understanding of the effects of the *APOE* genotype on normal brain function and its effects on neurodegenerative processes (Sullivan et al. 2004). We observed greatly impaired spatial learning in the doxorubicin-treated *APOE4* mice compared to the *APOE3* mice; indeed, the *APOE4* mice showed minimal learning over four training days. Control *APOE4* mice, both in the current and in prior studies, have reduced spatial learning compared to *APOE3* mice (Bour et al. 2008; Rodriguez et al. 2013), but not of the magnitude seen after exposure to chemotherapy. This is consistent with the differences seen in brain structure and function of *APOE4* human subjects (Caselli et al. 2004; Filippini et al. 2009). For instance, *APOE4* mice show decreased dendritic complexity (Dumanis et al. 2013; Rodriguez et al. 2013), and increased inflammation in response to amyloid pathology (Rodriguez et al. 2014) and other inflammatory stimuli (Zhu et al. 2012). *APOE* (Gafencu et al. 2007) and *APOE*-derived peptides (Christensen et al. 2011) have been linked to regulation of inflammatory pathways, which may have a role in the mechanism underlying cognitive-related cognitive decline. *APOE* is also associated with brain plasticity, and the *APOE4* genotype may confer an increased susceptibility to damage from chemotherapeutic agents as a result of altered neuroplasticity (Laws et al. 2002; Nathoo et al. 2003). Chemotherapy appears to accelerate aging, and shares inflammatory and plasticity pathways with aging (Mandelblatt et al. 2014a). Thus, the preclinical human *APOE* knock-in mouse model we have developed recapitulates human cancer-related cognitive decline in its clinical manifestations and structural features. For these reasons, this novel genetic preclinical model appears to be a valid and relevant preclinical platform to study the mechanisms of cognitive impairment at the intersection of aging, cancer, and genetic risk for neurodegeneration.

The major strengths of this properly powered in-vivo study include the integration of doxorubicin exposure with the use of both longitudinal behavioral analyses and ex-vivo brain structure assessment by VBM in the context of different human *APOE* genotypes. There are several caveats that should, however, be considered in evaluating our results. First, while single exposures to chemotherapy are common in preclinical models (Reiriz et al. 2006; Yadin et al. 1983; Yanovski et al. 1989), it will be important to test repeated doses of doxorubicin treatment to determine whether that paradigm confers increased risk of cancer-related cognitive decline. It will also be informative to assess the effects of additional treatments commonly used in the clinic, such as hormonal therapy, which exhibits independent effects on the brain. Second, with regard to neurological performance,

additional behavioral assays should be used in subsequent studies to define which cognitive domains are best suited for the analysis of specific regional brain impairments, such as attentional components that are affected in humans with cancer-related cognitive decline (Winocur et al. 2018). Third, tumor-naïve mice were used in order to reduce variability due to tumor burden and associated morbidity, which could affect the capability of the animals to complete tasks. Since cognitive impairment has been noted even before treatment in human cancer populations (Jansen et al. 2011; Wefel et al. 2004), it will be important to repeat our experiments in mice with cancer. Finally, these studies were performed on young animals for a proof-of-concept, and there is a clear need to assess the impact of aging on behavior and brain biology following chemotherapy.

Overall, we report poorer cognitive behavior as well as altered hippocampal and cortical volumes in chemotherapy-treated *APOE4* mice compared to *APOE3* mice and unexposed controls. These results are consistent with clinical studies of *APOE4* humans showing an increased risk for cognitive impairment after cancer chemotherapy. By generating the rational basis for the use of *APOE* status and age to inform treatment decision-making and survivorship care surveillance, this mouse model has direct implications for translation into clinical care in the growing population of cancer patients and survivors.

Acknowledgements

This research was supported by the NIH R35 CA197289 and R01 CA129769 to JM, R01 NS100704 to GWR, T32NS041231 to APS, T32CA009686 to TD, and a Georgetown University Medical Center Toulmin Pilot Grant to GWR and JM. This study was also supported in part by the NIH grant P30 CA51008 to Lombardi Comprehensive Cancer Center for support of the Animal Model and Preclinical Imaging Shared Resources for animal handling and imaging.

We would like to thank Idalia Cruz for her expertise with animal restraint and injection. The authors would also like to acknowledge Charrell Sherman for her assistance with multiple behavioral assays. Finally, we thank Dr. Amanda DiBattista for sharing her Barnes maze protocols and suggestions with our research team.

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Figure 1. Open field and elevated plus maze behaviors by *APOE* genotype related to doxorubicin exposure time.

(A,B) Open field locomotion was measured as the distance traveled over five minutes (in meters). (C,D) Exploratory behavior was measured as the number of rearing events in the open field (E,F). Anxiety was measured as the duration spent in the open arms of the elevated plus apparatus over 5 minutes. Measures were taken for untreated *APOE3* and *APOE4* mice (panels A,C,E), or for treated *APOE* mice one-week prior to exposure to doxorubicin, one-week post-exposure, and six weeks post-exposure (panels B,D,F). Doxorubicin-naïve *APOE* mice had no significant differences across *APOE* genotypes in locomotion, rearing, or anxiety between genotypes. Both treated *APOE* genotypes showed significantly decreased locomotion and exploration one-week post-exposure, while only the *APOE4* mice continued to show significant reductions from pre-treatment levels at six-weeks. There were no effects of doxorubicin on anxiety-related behavior. *APOE3*, n=7; *APOE4*, n=8; *APOE3*+dox, n=11; *APOE4*+dox, n=13. Measures are mean values $\pm$ SEM. 1-

way ANOVA with Tukey's HSD post-hoc correction for multiple comparisons. ** $p < .01$ *** $p < .001$ vs. pre-treatment.

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Figure 2. Barnes maze spatial learning and memory in untreated and doxorubicin-exposed *APOE3* and *APOE4* mice. Performance of untreated animals (panel A) and doxorubicin-exposed animals (panel C) throughout Barnes maze training days 1–4 (TD1–4) with inset for the four trials (T1–4) on training day 1. Reduction in latency to the target hole is recorded as a measure of training in this spatial learning task. Compared to *APOE4* mice, *APOE3* mice showed significantly shorter latencies during all four training days and during individual trials on training day 1, in both untreated (A) and treated (C) cohorts. A 72-hour probe trial for untreated animals (panel B) and doxorubicin-exposed animals (panel D) showed lower latency for *APOE3* mice compared to *APOE4* mice, irrespective of treatment. Data are shown as mean latency to complete the task $\pm$ SEM. Barnes maze: 1-way ANOVA with Tukey's HSD post-hoc correction for multiple comparisons. 72-hour probe trial: two-tailed student's t-test. (*APOE3*, n=7; *APOE4*, n=8; *APOE3*+dox, n=11; *APOE4*+dox, n=13) **p<.01 ***p<.001 *APOE4* vs. *APOE3* mice on the same trial or training day).

Figure 3. Barnes maze strategies to locate the escape target.

The strategy employed by each mouse to locate the target hole in each trial was recorded during training. (A) On the first training day (TD1), both untreated *APOE* genotypes used indirect exploration strategies (random activity or serial exploration of holes) to locate the escape hole. By the fourth training day (TD4), both *APOE3* and *APOE4* mice were employing a direct approach to the target hole in similar proportions (71% vs. 62%, respectively). (B) For doxorubicin-exposed animals, both *APOE* genotypes on the first TD used largely indirect search strategies. On TD3 and TD4, the *APOE3* mice treated with doxorubicin (*APOE3*+dox) used a direct method to locate the escape hole more often than the *APOE4*+dox mice (52% vs. 29%). There was a significant effect of *APOE* genotype in search strategy on TD3 for doxorubicin-naïve mice (Fisher's exact test of independence, $p=0.01$) and a significant effect of *APOE* genotype in search strategy on TD3 and TD4 for doxorubicin-exposed mice (* $p=0.01$ and $p=0.03$, respectively).

Figure 4. Effects of *APOE* genotype on *ex-vivo* brain MRI.

Voxel-based morphometry t-test analyses of brains of five to six month old control female *APOE3* mice (n=6) and *APOE4* mice (n=6) mice (A,B) or doxorubicin-treated *APOE3* mice (n=5) and doxorubicin-treated *APOE4* mice (n=6) (C,D). Maximum intensity projection of the VBM results in sagittal, coronal and axial slices and the design of the matrix for the study comparing control *APOE3* versus control *APOE4* mouse brains (A) or doxorubicin-treated *APOE3* versus doxorubicin-treated *APOE4* mice (C). Colored overlays corresponding to Panels A and C on a co-registered template image showing the location of significant clusters in control *APOE3* versus control *APOE4* mouse brains (B) or doxorubicin-treated *APOE3* versus doxorubicin-treated *APOE4* mice (D). The color bar shows the T-values of the statistical analyses. VBM performed across the entire brain revealed significantly more GM, defined by red areas (p<0.01).

Figure 5. Effects of doxorubicin treatment on *ex-vivo* brain MRI.

Voxel-based morphometry analyses of 3-D anatomical MR images of doxorubicin-treated *APOE3* and *APOE4* mice versus control *APOE3* and *APOE4* mice, respectively. Maximum intensity projection and pseudo-colored images of F-test clusters depicting regional brain differences between control versus doxorubicin-treated *APOE3* mouse brains (A and C), and control versus doxorubicin-treated *APOE4* mouse brains (B and D). Maximal differences in GM volumes using SPMMouse are shown in red color ($p < 0.03$).

Table 1.

Percentages of the allotted time used for Barnes maze completion across the four training days (TD).

Treatment Groups	TD1	TD2	TD3	TD4
<i>APOE3</i>	50%	25%	12%	7%
<i>APOE3</i> + doxorubicin	55%	26%	15%	9%
<i>APOE4</i>	83%	47%	29%	12%
<i>APOE4</i> + doxorubicin	88%	78%	71%	55%