THE EFFECT OF ESTROGEN ON MICROGLIAL INFLAMMATION IN
SURGICALLY OVARIECTOMIZED MONKEYS

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Introduction

Menopause

Menopause is a natural aging process in women which affects some metabolic and physiological states by reducing the gonadal steroid hormonal levels (Lei et al., 2003). Hormonal changes bring a series of consequences including, but not limited to, hot flashes, night sweats, fatigue, decreased libido, and mood changes (Al-Musa et al., 2017). These mood changes may include depression, irritability and emotional liability (Al-Musa et al., 2017).

Health Consequences of Menopause

In some cases, menopausal women can be prone to more serious health risks such as osteoporosis (Yoldemir, Erenus & Durmusoglu, 2012), cardiovascular diseases (Pei et al., 2017), and neurocognitive impairments such as Alzheimer’s disease (Roos, 2012) due to loss of estrogen. The major predictor of menopause-induced osteoporosis in women is reduced bone mineral density with no previous history of fracture (Yoldemir, Erenus & Durmusoglu, 2012). Reduced absorption of calcium in kidneys and intestine leads to this bone density decrease (Dabirnia, Mahmazi, Taromchi, Nikzad & Saburi, 2016). About 50% of women above the age of 50 experience an osteoporosis fracture following menopause (De Vos, Devroey & Fauser, 2010). Hormone replacement therapy (HRT) can appear effective in the process of healing osteoporosis (Mosekilde, Nielson et al.,
2000). However, menopause is not the only factor inducing osteoporosis and other factors may affect bone density in women. Additionally, estrogen in premenopausal women has a protective role against hypertension-induced ventricular remodeling (Pei et al., 2017). Therefore, going through menopause puts women at a much higher risk to experience cardiovascular diseases following reduction in estrogen levels. HRT seems to help alleviate the symptoms of Coronary Artery Disease (Henderson, Paganini & Ross, 1991).

Although employing HRT as a first line treatment seems like a rational solution to prevent osteoporosis in post-menopausal women, results of Women Health initiative study (WHI) and Million Women’s Study primarily suggested negative effects of HRT on the human subjects (Gambacciani & Levancini, 2014). These safety concerns, however, have been mainly revised by the international medical associations and HRT is now again utilized to prevent osteoporosis (Gambacciani & Levancini, 2014). These revisions suggest that as long as HRT is used to treat women younger than 60 years old or within 10 years after menopause, it should appear effective and a good approach in preventing osteoporosis (Gambacciani & Levancini, 2014). Additionally, more recent data of the WHI study indicate that specifically estrogen replacement therapy (ERT) should not persist for more than three-four years due to adverse effects on the cardiovascular system as well as triggering breast cancer (Checa et al., 2005). The type of HRT is another factor to take into account in preventing osteoporosis. For instance, a combination of estrogen and progesterone should be given to women with an intact uterus, while estrogen without progesterone to hysterectomized women (Checa et al., 2005). According to another study, introducing an additive therapy that includes
etidronate disodium to HRT increases the bone mass density in women who have low bone mass or show no response to HRT (Morishige et al., 2002).

**Brain Health**

The natural aging process in menopausal women can make the brain more susceptible to some cognitive impairments (Roos, 2012). One of the roles of estrogen has been shown to be enhancing learning and memory, particularly, working memory on spatial tasks (Dohanich, 2003). Working memory, is a type of short term memory, storing information for very brief periods of time (Dohanich, 2003). One of the most common forms of dementia in aged populations is Alzheimer’s disease (AD) (Rosalind, Bai & Bai, 2017). AD, which affects about 10% of the over 65-year-old population, becomes more susceptible with aging (Rosalind, Bai & Bai, 2017). This neurodegenerative disorder takes place in three stages (Rosalind, Bai & Bai, 2017). In the early stage, the patient shows symptoms of recent memory loss and personality changes (Rosalind, Bai & Bai, 2017). In the mild-moderate stage, the patient starts experiencing more consistent and pervasive memory losses and some mobility and coordination dysfunctions (Rosalind, Bai & Bai, 2017). In the severe stage, patients experience severe past memory loss and are generally confused about the present (Rosalind, Bai & Bai, 2017). They also completely lose their verbal skills. (Rosalind, Bai & Bai, 2017). Preliminary studies suggest that HRT can reduce the risk of these cognitive impairments (Maki, Zonderman & Resnick, 2001). However, a controversial Women’s Health Initiative Memory Study (WHIMS) suggested that HRT actually worsens the cognitive impairments in post-
menopausal women (Zhang et al., 2016). Due to these conflicting findings, our study was
designed to shed more light on the role of HRT in post-menopausal cognitive changes.

**Common Imaging Techniques**

Magnetic Resonance Imaging (MRI) Positron Emission Tomography (PET)
techniques are commonly used to visualize cognitive changes. In one of these studies,
MRI imaging was adopted to trace the effects of HRT on the deep white matter
hyperintensity and periventricular hyperintensity in the brains of healthy post-
menopausal women (Liu et al., 2009). Likewise, MRI and PET were used to find the
association between hippocampal volume and verbal memory skills in a sex-based study
on Alzheimer’s Disease (Caldwell, Berg, Cummings & Banks, 2017). There are a wide
variety of studies on the brain that take advantage of these imaging methods to measure
the metabolic and physiological processes of the brain.

**WHIMS**

On the other hand, the results of a study done by Women’s Health Initiative
Memory Study (WHIMS) revealed negative impact of HRT on the brain (Zhang et al.,
2016). According to this study, conjugated equine estrogen known as Premarin, both
paired and in the absence of synthetic medroxyprogesterone acetate (MPA) also known
as Provera, indicated detrimental effects on the cognition of women aged 65 or older
(Zhang et al., 2016). These adverse effects included increased risk of dementia (Zhang et
al., 2016). Additionally, MRI imaging of the brain demonstrated less medial prefrontal
grey matter and decreased volume of hippocampus (Zhang et al., 2016). One explanation
for the obtained results by the WHIMS study can be the time interval between the occurrence of menopause and beginning of HRT in the study subjects (Voytko, Tinkler, Browne & Tobin, 2009). The age of the participants as well as their pathological state may have been other factors leading to the negative results (Voytko et al, 2009). Another rationale behind these negative effects can be the type of HRT used in these studies. The artificially synthesized Premarin and Prempro (Premarin-Provera complex) might not have appeared as effective as 17β estradiol and progesterone used in the previous HRT studies.

*Animal Models*

Cognitive changes in response to HRT or in the absence of it have been widely studied on animal models. Non-human primates and rodents are the most popular animal models for studies on menopause (Koebele & Bilmonte-Nelson, 2016). Although, non-human primate models resemble humans in their menstrual cycle more closely than rodents, they are costlier to study (Koebele & Bilmonte-Nelson, 2016) and have a much longer life span compared to 2-3 year life span of rodent models. Furthermore, rodent models do display similar hormonal changes and cognitive alterations to humans (Koebele & Bilmonte-Nelson, 2016). For example, they respond to decreased levels of luteinizing hormone (LH) followed by decreased estradiol and progesterone levels by altering their normal estrous cycle, similar to human menstrual cycle (Koebele & Bilmonte-Nelson, 2016). HRT in OVX rodent models has resulted in spatial memory enhancement as measured by maze studies (Koebele & Bilmonte-Nelson, 2016). Also, combining estrogen and progesterone has been proven to enhance the performance of
middle-aged rodents on radial-arm maze (assessment of reference and working memory) study (Koebele & Bilmonte-Nelson, 2016). Ovariectomy of rodents significantly reduces their memory and learning skills as indicated by the results of the Morris water maze test (Kaur, Jindal, Kaur & Chopra, 2012). These cognitive deteriorations were linked to the great reduction in circulating estrogen and progesterone in the ovariectomized rodents (Kaur, Jindal, Kaur & Chopra, 2012). According to other in vitro studies, HRT including 17ß estradiol or raloxifene can prevent the inflammatory response in the brain of rodent models by changing the expression of microglial cells and astrocytes (Mouton et al., 2002). Specifically, treating ovariectomized mice with 17ß estradiol and raloxifene decreased the number of microglial cells and astrocytes in the dentate gyrus and CA1 region of the hippocampus (Mouton et al., 2002).

The inflammatory response of microglial cells is one of the major hallmarks of AD. The mechanism leading to AD involves accumulation of amyloid-beta deposits in the brain forming plaques, which in turn, cause an inflammatory response by microglial cells (Coman et al., 2017). Inflamed microglia then increase the secretion of cytokines. Other studies have shown that estrogen replacement therapy can, to some extent, lower the risk of these neurocognitive impairments and prevent microglial inflammation. Therefore, the focus of this study was primarily on microglia of the hippocampus.

**Non-Human Primate Models**

Non-human primate models have been especially useful in understanding the aging processes following by menopause. Using non-human primate models provides a number of advantages that include but are not limited to the ability to study visual non-
spatial cognitive processes and the ability to operate similar behavioral tasks to those used in human studies (Tinkler & Voytko, 2004). According to Tinkler and Voytko (2004), monkey models show similarities in cognitive profiles and the way they respond to hormonal changes and HRT, as well as their menstrual cycle to those of their human counterparts. Particularly, visuospatial attention function and visual memory are affected in monkey models (Tinkler & Voytko, 2009). However, non-human primates display some differences from their human counterparts, which limit our ability to generalize the results of the primate studies to human population (Tinkler & Voytko, 2004).

Experimental models are thus used to study these processes. For example, some of the aging processes are not naturally observed in non-human primates (Tinkler & Voytko, 2004). These animals have played a significant role in discovering some of the most important substrates involved in aging (Tinkler & Voytko, 2004).

**Our Study**

In this study, tissue from OVX and ERT treated post-menopausal monkeys were used to analyze brain inflammatory response by quantifying morphologically distinct microglial cells in the hippocampus. Specifically, we hypothesized that loss of circulating estrogen in the OVX group would lead to a high number of activated microglial cells, while in the ERT group, this number would be smaller indicating a lower inflammatory response.
Methods

Animal subjects and tissue preparation

Brains were obtained thanks to a generous donation of banked tissue from Mary Lou Voytko, Ph.D. All procedures involving these animals were conducted in compliance with state and federal laws, standards of the United States Department of Health and Human Services, and guidelines established by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee. Brains from sixteen female cynomolgous monkeys (Macaca fascicularis, age estimated by dentition at approximately 18-23 years) were donated from a previous study that assessed the effects of OVX and ERT on various physiological parameters (Lees et al., 2007).

Six normally cycling sham-operated intact control monkey brains were available for this study. In addition, 11 animals were ovariectomized and given either a placebo or estrogen treatment for six months. Ovariectomies had been performed at the Wake Forest University School of Medicine under appropriate anesthesia. Estrogen replacement therapy (ERT n=5, conjugated equine estrogens, aka Premarin, Wyeth-Ayerst, Radnor, PA) was administered orally at a dose of 21 µg/kg daily to approximate the standard dose of daily 0.625 mg Premarin prescribed to postmenopausal women. Ovariectomized monkeys receiving placebo (n=5) were given the vehicle suspension with no conjugated equine estrogens.
After six months, the animals were restrained with ketamine (15 mg/kg i.m.) and deeply anesthetized with sodium pentobarbital (35 mg/kg i.v.), and perfused with ice-cold lactated Ringers or phosphate buffered saline, and a bone saw was used to gain rapid access to the brain. Brains were removed, hemisected, blocked coronally, and the left hemisphere was immediately immersion-fixed in 4% paraformaldehyde for two weeks. Necropsy and fixation protocols were performed by the same individual and kept uniform from animal to animal. Brains were then cryoprotected in a series of phosphate-buffered sucrose and frozen at -80° C. Serial sectioning of brain blocks containing the hippocampus (temporal and parietal blocks) was performed at 50 µm on a freezing sliding microtome. Sections were collected and stored at -20° C in freezer storage solution until all tissue was available for immunoprocessing.

**Immunohistochemistry**

Using standard immunohistochemical techniques, a 1-in-24 series of sections in each animal was processed for Iba-1 protein. The Iba-1 protein is found on microglia cell membranes, making it a specific marker for microglia (Ohsawa et al, 2004). Tissue from each group was represented in each immunohistochemical run. Briefly, sections were rinsed in 0.1 M phosphate-buffered saline (PBS, pH = 7.4) ten times for 5 minutes per rinse to remove freezer storage solution. Sections were then incubated at 90° C for 30 minutes in a citriconic acid (pH 7.4) solution to unmask antigen binding sites. After incubation, endogenous peroxidases were quenched in 75% methanol/2.5% hydrogen peroxide for 20 minutes. Tissue was then blocked for one hour in a solution containing 4% normal goat serum, 5% bovine serum albumin, and 0.6% Triton X-100 to prevent
nonspecific binding of primary antibody. Following the blocking step, tissue was incubated in primary antisera (Iba1 rabbit polyclonal, diluted 1:10,000 in PBS, Wako Laboratory Chemicals, Richmond, VA) for 48 hours at -4\°C on an orbital shaker. The sections were then rinsed and incubated in a biotinylated secondary antibody solution (0.5% goat anti-rabbit, Vector, Burlingame, CA) for 1 hour, rinsed in PBS, and incubated in avidin-biotin-complexed horseradish peroxidase (ABC Elite, Vector) for 1 hour. Sections were again rinsed, then reacted with the chromogen 3,3’-diaminobenzidine (DAB) in 0.002% H\textsubscript{2}O\textsubscript{2} to catalyze the reaction (DAB substrate kit, Vector). Nickel sulfate was used to create a blue-black reaction product. Sections were rinsed, mounted, cleared, and coverslipped with DPX.

**Stereological Analysis**

Unbiased stereological methods were used to quantify numbers of Iba-1 positive microglia in the hippocampus. Regions were outlined at 10x and microglia were counted at 63x on a Leica DM2500 microscope. Live images were analyzed with Stereologer Software (Stereology Resource Center, Baltimore, MD).

Region volume was obtained using Stereologer’s Cavalieri Volume estimator. The first available section through the hippocampus was taken as a random starting point, and cells were counted on every 24th section. Stereologer electronically generated a counting grid (1200 x 1200 µm), and cells were counted at each grid point using the optical fractionator variation of the optical dissector (West, 1993). Standard counting rules were applied to a 100x100x10 µm counting frame with a guard zone of 2 µm.
To further understand the nature of microglia inflammation in the brain, microglia were divided into three main subtypes based upon morphology using established methodology. Briefly, ramified microglia are resting microglia that appear as a small, round or rod-shaped cell body with long, highly arborized/branching processes. As microglia become more active, they retract processes and enter intermediate stages of morphology, characterized by fewer processes that possess fewer branch points. Amoeboid microglia are the most active and most retracted, displaying cell bodies that have minimal or no processes, potentially a ruffled border, and may be hypertrophic. Sampling schemes were designed to obtain a coefficient of error (CE) of 0.1 or less for both ramified and intermediate microglia cell types. Amoeboid microglia were rare, and thus CE remained at approximately 0.15. Data was collected with experimenters blind to animal condition.

**Statistical analyses**

SigmaStat software was used to perform all statistical calculations. One-way Analysis of Variance (ANOVA) was used to test for significant effects of total microglia number and density between the groups, and two-way repeated measures ANOVA was used to examine group x microglia type interactions, where microglia type was the repeated-measures factor.
Results

Immunohistochemistry for Iba-1 yielded intensely stained microglia, with labeling present uniformly throughout the soma and processes (Figure 2). Control sections omitting the primary antibody or secondary antibody did not display any reactivity following exposure to the chromogen DAB. There were no obvious qualitative differences in microglia staining intensity, number, or density between the groups, and uniform dense fields of microglia under magnification necessitated quantitative analysis.

Microglia number and staging

Stereological analysis indicated that hippocampal region volumes were not statistically different between the groups (F (2,15) = 0.0756, P = 0.928). There were no treatment-based differences in estimated total microglia number (F (2,15) = 0.651, P = 0.538; Figure 3) or microglia density (F (2,15) = 2.877, P = .092; Figure 4).

As expected, there was a significant main effect of Microglia Type (F (2,47) = 140.773, P < 0.001), indicating that the subjects had differing amounts of ramified, intermediate, and amoeboid microglia, with ramified being the most prevalent category and amoeboid being relatively rare by comparison (Figure 5). There was no main effect of Treatment Group nor Treatment Group x Microglia Type interactions for either estimated total numbers (F (4,47) = 1.327, P = 0.286; Figure 5) or densities (F (4,47) = 2.019, P = 0.121; Figure 6).
Discussion

This study investigated the effects of estrogen loss and replacement on microglial inflammation using a non-human primate model. The three study groups consisted of the intact, normally cycling estrogen monkeys, monkeys ovariectomized for 6 months (OVX), and ovariectomized monkeys treated with estrogen for 6 months (ERT). We did not find significant differences in the hippocampal volume among the three groups. Additionally, the total number or density of microglia did not differ significantly among the treatment groups, although there was a nonsignificant trend for microglia density to be lower in both OVX and ERT treated groups, as opposed to Intact controls.

As it is possible that changes to hormone status increase inflammation not by altering total microglia but rather shifting the existing microglia to a more activated form, we also divided our results into three broad categories of inflammation, as used elsewhere. Again, no significant differences were found.

A number of other studies have focused on the aging of brain cells and its relation to microglial inflammation and ovarian hormonal levels. One of the hallmarks of brain aging is the chronic inflammation of microglial cells (Cribbs et al., 2012; Raj et al., 2015). A recent study confirmed previous research by showing that microglial activation increases with brain aging in the white matter pathways of frontal lobe, including the cingulate bundle, corpus callosum, and frontal white matter (Shobin et al., 2017). In other words, their results indicated that hypertrophic and amoeboid cells can be predictive of
the cognitive decline associated with aging (Shobin et al., 2017). While we did not find any significant alterations to the total number of microglia as well as microglial density, we did find a trend indicating a lower microglial density in the ovariectomized monkeys and ERT monkeys compared to the intact monkeys.

According to another study conducted on aged rhesus monkeys, long-term estrogen replacement therapy reversed the age-related cognitive impairments mediated by the dorsolateral cortex in OVX monkeys (Young, Ohm, Dumitriu, Rapp & Morrison, 2007). Ovariectomy in aged monkeys seemed to reduce the multisynaptic boutons, which are directly involved in working memory performance (Hara et al., 2016). Using HRT that included cycling estradiol seems to restore the multisynaptic boutons in the surgically OVX monkeys (Hara et al., 2016). Finally, 17ß estradiol appeared to be effective in preventing the spatial memory and visuospatial attention task impairments from occurring in middle-aged OVX monkeys (Tinkler & Voytko, 2005). These studies and others suggest that HRT can be used to alleviate some of the post-menopausal complications as well as prevent the cognitive decline triggered by loss of circulating ovarian hormones. Our results suggested that inflammatory mechanisms may not be involved in these changes.

Women undergoing menopause are more susceptible to cognitive impairments that are closely associated with circulating estrogen. Using non-human primate models has provided a valuable opportunity to study the cognitive alterations following menopause. Particularly, surgically OVX monkey models are used due to their similar menstrual cycle and response to ovarian hormonal changes to humans. The aim of our
study was to use monkey models in order to provide more insight on ovarian hormonal levels and the associated immune response in the brain. The results of this study, though not significant, indicated a trend between the microglial cell type and HRT. This raises the question whether HRT can lower the microglial inflammation under different study conditions.

This study included a number of limitations involving the age of the models, their blood estrogen levels, and statistical variabilities. One limitation of our study is the duration of estrogen loss and replacement. If we had used a longer treatment period, we may have been able to see significant results, as it is possible that our trend at 6 months is merely a snapshot into gradually increasing inflammation. Additionally, the statistical analysis demonstrated high variability in the study groups. A total of 16 monkeys were analyzed for this study. With a larger sample size, we may have been able to find statistically significant differences that overcame this variability. Another group of monkeys undergoing 24-month treatment was excluded from the study due to time constraints. With the addition of this group, the variability seen within the groups may be reduced. Finally, the conclusions about the brain inflammatory response in this study were based on microglia morphology. It is possible that using immunohistochemical techniques to visualize microglial cells is not the strongest measure of the brain’s inflammatory response to ovariectomy. Overall, this study could have resulted in more significant results under modified conditions.

There are still so many unknowns about the HRT effects on brain’s immune response. The OVX and ERT groups demonstrated a trend toward lower microglial
densities compared to the intact group. This can be explained by the type of ERT used in our study, Premarin, which is a mixture of at least 11 chemically distinct estrogens or metabolites. It is possible that the function of the naturally occurring 17ß estradiol in reducing the microglial inflammation was disrupted through competitive inhibition with other estrogen types. An important modification that should be considered for future studies is to have two distinct HRTs given to the experimental groups. One ovariectomized group should receive 17ß estradiol and the other should receive Premarin. This may reduce the likelihood of the HRT confounding the inflammatory results.

An additional explanation for the trend toward lower hippocampal densities of the OVX and ERT groups may be related to the role of progesterone in the brain’s inflammatory response. Progesterone has been shown to have a neuroprotective role in some cases, such as those following a stroke (Won, Lee & Stein, 2015). In addition, progesterone may play a role in the functioning of the hippocampus and forebrain, thus, it may be associated with memory (Roozbehi, Sharafi, Karimi & Kamali, 2017). Previously, progesterone has been related to prevention of the neurodegeneration of the hippocampus (He, Yang, Zhai, Shao, & Li, 2011). Research supports the role of progesterone in improving memory tasks and cognition in rats (Roozbehi, Sharafi, Karimi & Kamali, 2017). The ERT and OVX groups both lacked progesterone, which wasn’t part of their ERT, while the intact group had normally cycling progesterone. Won, Lee and Stein (2015) showed that progesterone can attenuate the microglial inflammation induced by the tissue plasminogen activator in rats both in vivo and in vitro. Furthermore, the 17ß estradiol receptor complex seems to activate the endogenous progesterone
receptors in the brain that are involved in the normal activation of microglial cells in rats (Lee & Gorski, 1996). Additional studies could include two experimental groups, one receiving a combination of estrogen and progesterone, and the other only receiving estrogen.

Finally, the effect of estrogen on microglial inflammation can be further studied with a focus on the role of estrogen in reducing inflammation through a norepinephrine-mediated mechanism. It’s been shown that norepinephrine has an inhibitory role in the inflammatory response of the brain (Heneka et al., 2009). Estrogen receptors, ER-α and ER-β, increase the electrical excitability (Kaba, Saito, Otsuka, Seto & Kawakami, 1983). This in turn enhances the norepinephrine turnover rates in the hypothalamic areas (Wise, Rance & Barraclough, 1981). Eventually, this leads to increased release of norepinephrine from the mediobasal hypothalamus (Pau et al., 2000). The aim of all of these studies is to shed some light on the mechanisms involved in post-menopausal cognitive complications and develop strategies to prevent or treat these complications.
Figure 1. Hippocampus area of a cynomolgous monkey’s brain
Figure 2. Microglia morphology. A. Ramified microglial cell, B. Intermediate (hypertrophic) microglial cell, C. Amoeboid (activated) microglial cell
Figure 3. Microglial density: This figure describes the microglial density in terms of the number of microglia counted per cubic micrometer of the hippocampal region in the three monkey groups.
Figure 4. Microglia count: The average total number of microglia in the hippocampal region of the three monkey groups using stereological methods.
Figure 5. Microglia Type Number: The estimated average number of each morphological type of microglia counted in all the monkey models
Figure 6. Microglia Type Density: The estimated microglial density of each morphological type of microglia in the monkey brains.
References


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of Postmenopausal Women Revealed by Optimally-Discriminative Voxel-Based Morphometry. *Plos One, 11*(3). doi:10.1371/journal.pone.0150834
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
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<tr>
<td>OVX</td>
<td>Ovariectomized</td>
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<tr>
<td>ERT</td>
<td>Estrogen Replacement Therapy</td>
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<tr>
<td>WHI</td>
<td>Women's Health Initiative</td>
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<td>WHIMS</td>
<td>Women's Health Initiative Memory Study</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
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Table 1. Table of Acronyms