**Research Strategy**

**Background**

Progressive impairment of memory and cognition is a key clinical feature of **Alzheimer’s disease (AD**), which is characterized by extracellular **amyloid β-protein (Aβ)** deposits in the brain (plaques), intraneuronal tau pathology, neuronal cell death, and inflammation that ultimately manifest in the form of neuropsychiatric symptoms including depression and anxiety (Galts et al., 2019; Mendez, 2021). Around 95% of hospitalized patients with AD have the sporadic form of disease known as late-onset AD (Diniz et al., 2017). No effective cure for AD has been established, highlighting the need to identify novel compounds that can counteract the AD course.

Dementia is more than twice as common in women than in men, even in middle age (Chêne et al., 2015; Koran et al., 2017). Estrogen regulates key processes implicated in AD pathogenesis, in particular β-amyloid protein accumulation (Scheyer et al 2018). Understanding the biology of sex-related differences in cognitive function will not only provide a framework to prevent AD, but is also integral to the development of personalized, sex-specific medicine (Li and Singh, 2014).

A fundamental challenge to the treatment of AD is that its accurate diagnosis relies on clinical criteria and the presence of symptoms such as memory loss and cognitive difficulties (Sabbagh et al., 2017). In the search for better biomarkers, epigenetic modifications have emerged as important players in the development of AD, with potential implications for its treatment (Perkovic et al., 2021). **MicroRNAs (miRNAs(** are short noncoding RNAs that modulate gene expression and are closely linked to AD pathogenesis (Kou et al., 2020). Importantly, miRNA expression profiles in AD patients are distinct from those of healthy controls (Perkovic et al., 2021), suggesting a possible role for these miRNAs as novel biomarkers and/or therapeutic targets in AD. miRNAs can be detected in the peripheral circulation or in the brain tissue, and several miRNAs (e.g. miR-9, miR-29abc, miR-34a, miR-107, miR-125b, miR-132, miR-146a, miR-155) have shown promise as AD biomarkers in the periphery and the central nervous system (CNS) (Perkovic et al., 2021). However, the mechanisms underlying how miRNAs may slow neurodegenerative processes are largely unknown.

The hippocampus-prefrontal cortex (**HPC-PFC**) pathway plays a fundamental role in executive and emotional functions. Disruptions in HPC-PFC functional connectivity can contribute to neuropsychiatric symptoms observed in mental illnesses and neurological conditions, such as AD, depression, and anxiety disorders (Kovner et al., 2019; Ruggiero et al., 2021). Exposing hippocampal and cortical neurons to Aβ peptide activates glycogen synthase kinase 3β (GSK-3β) and thereby drives Wnt/β-catenin signaling pathway degradation, contributing to neurofibrillary tangle formation and impaired neuronal survival. The gene encoding the Dicer protein, which regulates miRNA maturation, is a key β-catenin target, thus outlining a mechanism whereby AD can directly promote miRNA dysregulation through the Wnt signaling pathway. Altered miRNA expression has consistently been observed in cerebrospinal fluid (CSD) samples from AD and depression patients and in the hippocampal tissue of model animals (Bisrat et al 2016; Teo and Soga, 2018 Chopra et al 2020; Muller et al 2014’ Walgrave et al 2021).

**Cannabidiol (CBD)** is a safe, non-psychoactive phytocannabinoid that reportedly exhibits immunomodulatory activity in neurodegenerative disease, and may ameliorate the symptoms of AD and slow cognitive decline (Li et al., 2020). CBD can promote PI3K/Akt signaling, which in turn inhibits GSK-3β, thus increasing Wnt/β-catenin pathway activity and exerting neuroprotective activity against Aβ-induced neurotoxicity in AD (Cassano et al., 2020). Consistenetly, in an *in vitro* model of AD, CBD treatment suppressed the hyperphosphorylation of tau protein-mediated to β-catenin and GSK-3β, in Aβ-stimulated PC12 neuronal cells (Esposito et al., 2006). Moreover, CBD decreased Aβ levels in SH-SY5Y cells transfected with the amyloid precursor protein (SH-SY5YAPP+) (Scuderi et al., 2014), and, in a mouse model, CBD administration ameliorated cognitive impairment (Cheng et al., 2014). In one recent study, CBD enhanced the expression of interleukin (IL)-33 and TREM2 (triggering receptor expressed on myeloid cells 2), which were associated with improved cognitive function and reduced AD symptoms (Khodadadi et al., 2021).

Given the urgent need to develop new disease-modifying medications that can mitigate AD progression, we propose the present study with the aim of improving the early diagnosis and treatment of this devastating disease. Specifically, in our planned studies, we propose to examine AD-related shifts in miRNA expression profiles, regulatory mechanisms in the HPC-PFC pathway, male and females rats, AD-induced alterations in miRNAs associated with AD and related regulatory mechanisms in the HPC-PFC pathway and to provide novel therapies against AD in the short- (CBD) and long-term (miRNAs).

Together, our planned experiments will offer insight into the potential therapeutic utility of the targeted activation or silencing of specific miRNAs as an approach to restoring memory and alleviating emotional deficits, while also better defining the role that miRNAs play in the context of the CBD-mediated treatment of AD in both males and females, highlighting a mechanistic basis for the slowing of neurodegenerative processes. Ultimately, the establishment of a validated noninvasive biomarker of AD or associated targets will guide the future development of early diagnostic tools, preventive strategies, and effective pharmacological treatments for dementia.

**Significance**

AD is the world's leading cause of dementia and the global AD patient population continues to grow. However, none of the available treatments for AD prevent or reverse the progression of this disease, instead treating its symptoms with limited efficacy and the potential for adverse effects (Salomone et al., 2012). Defining non-invasive, readily accessible biomarkers amenable to early AD diagnosis and effective disease-modifying treatments is thus of critical importance.

CBD holds promise as a potential clinically safe and efficacious disease-modifying therapy that may attenuate neurocognitive decline and emotional dysfunction. Findings from this study will offer insight into whether CBD can exert these neuroprotective benefits through a bi-directional dialogue with miRNAs and Wnt/β-catenin signaling. A drug that acts on a single pathway is unlikely to diminish the complex pathological cascade that ultimately leads to AD (Watt and Karl, 2017). CBD has a wide range of targets, suggesting it may have potential as a multimodal treatment for AD. Our findings will be well-suited to rapid clinical translation given that CBD is readily available, appears to only have limited side effects (Bergamaschi et al., 2011) and is safe for human use (Leweke et al., 2012). Our study will provide proof of principle for the validity of CBD as a candidate treatment for this devastating disease.

miRNAs regulate a diverse array of AD-related processes and may thus offer wider-ranging benefits than the available treatments. miRNAs are interesting therapeutic targets given their ability to regulate endogenous gene expression such that one miRNA can potentially regulate entire biological pathways. As such, miRNA-based therapeutic strategies may be ideally suited to AD given its complex etiology. These therapeutic effects may be achieved by completely or partially ablating the functions of miRNAs of interest in order to downregulate the expression of targeted genes and proteins involved in disease pathogenesis. By identifying miRNA biomarkers associated with AD, our study will define non-invasive biomarkers that may aid in the early diagnosis of this disease such that appropriate treatments can be administered prior to the onset of irreversible dementia. While the miRNA therapeutic strategies employed in our animal models are too invasive for human implementation, this work will provide an invaluable and unprecedented framework for AD treatment by identifying specific miRNAs that can be delivered through a safer route.

In many cases, drugs are used in clinical settings without a full understanding of the molecular mechanisms through which they function. Understanding the mechanism of action for a given drug in greater detail has the potential to support further pharmacological development efforts and to mitigate the risk of failed clinical trials by stratifying patients to focus on subpopulations most likely to respond to such treatment. CBD may exert its therapeutic efficacy at least in part by interacting with miRNAs. By simultaneously clarifying the specific miRNAs that predict disease and the miRNA-mediated mechanisms underlying the neuroprotective benefits of CBD, our study may suggest that a combination of both miRNA-based and CBD-based therapeutic interventions may lead to superior therapeutic outcomes, transforming the clinical and therapeutic AD space.

**Innovation**

The early diagnosis of AD will enhance our understanding of the pathological mechanisms underlying this disease, and will support the development of innovative preventive strategies. As there is no effective cure for AD, and the treatments available can only reduce the symptoms in the initial phase of the disease, it is of paramount importance to identify novel compounds that can treat this disease and prevent its progression. Our work will refine existing approaches and has the potential to significantly impact the early diagnosis of AD, clarify its pathophysiological mechanisms, and support the development of interventional strategies in the short- and long-term. CBD treatment can be immediately translated to humans to relieve symptoms whiel further studies of the clinical manipulation of miRNA activity progresses.

**Approach**

**Aim 1:** **To identify AD-related patterns of miRNA, mRNA, and protein dysregulation that may be reversed by CBD treatment, and to explore the utility of peripheral miRNAs and inflammatory cytokines as biomarkers of AD progression and treatment responses.**

Studies completed to data have identified several miRNAs implicated in the regulation of dementia-related proteins and pathways including miR-9, miR-29abc, and miR-34a (For a recent review see Perkovic et al., 2021). However, the molecular and cellular mechanisms underlying how these miRNAs may slow neurodegenerative processes are largely unknown. To address this gap in knowledge, In our first experiment, will be administered in a streptozotocin (**STZ**)-induced rat model of sporadic AD, after which cognitive and emotional function correlations with alterations in the expression of miRNAs in the HPC-PFC pathway, as well as targets related to inflammation, CBD signaling, AD pathology, and β-catenin will be assessed. Briefly, male and female adult and middle-aged rats will receive intracerebroventricular (ICV) injections of STZ, as this is a widely used method for modeling neuroinflammation and neurodegenerative processes. We have successfully established this model in our laboratory and have generated preliminary data demonstrating the impaired performance of STZ adult male and female rats in object recognition and object location tasks (Figure 1). These STZ rats are treated with vehicle or CBD and their cognitive and emotional function will be assessed. Preliminary data from our lab in female naïve middle-aged (15 months) rats suggest that CBD can be beneficial as a means of improving emotional function and memory performance (Figure 2).

*Add figure 2 from Nadya: CBD may be beneficial for the elderly*

*Add figure 1 from Shira: Impaired performance in STZ rats in the object recognition and object location tasks*

Cognitive and emotional function will be correlated with alterations in the expression of **miRNAs in the HPC-PFC pathway** and several interacting systems and targets including **inflammatory markers** [inducible nitric oxide synthase (iNOS), glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba-1), and arginase-1 (ARG-1)], **primary targets of CBD** [including the CB1 and CB2 receptors, FAAH (fatty acid amide hydrolase), which hydrolyzes the endocannabinoid anandamide, and the serotonergic receptor 5HT1a], **markers associated with AD pathology** [Aβ protein, phosphorylated Tau (p-Tau Ser396) protein], and **β-catenin**. We will also investigate **peripheral miRNAs as potential biomarkers** of AD and treatment responses and **pro-inflammatory cytokines** (IL-1β, IL-6, and TNF-α) in whole blood samples and correlate these alterations with the ones observed in the brain. Findings from this initial experiment will clarify AD-related gene regulation and the degree to which such dysregulation is reversed by CBD treatment. Male and female STZ rats may exhibit similar cognitive and emotional patterns of dysfunction while also exhibiting distinct sex- and brain region-dependent changes in the expression of miRNAs in response to STZ and to CBD treatment (Manolli and Tollkuhn, 2018). Accordingly, studying both male and female animals will be essential to gain full insight into sex-specific patterns of AD- and CBD-related miRNA regulatory activity. Peripheral miRNAs also have the potential to be used as biomarkers of AD and to predict therapeutic responses. Our preliminary data suggest that CBD reverses stress-induced increases in miR-16 and miR-135 levels in the medial PFC in an unpredictable chronic mild stress (UCMS) rat depression model (Figure 3).

*figure 3 from Uri demonstrating that CBD normalized stress- induced increase in miR-16 and miR-135 levels in the medial PFC in a rat model for depression*.

CBD normalized UCMS- induced increase in miR-16 and miR-135a levels in the mPFC. Rats exposed to UCMS + Veh demonstrated increased miR-16 and miR-135a expression compared to No UCMS-Veh (p<0.001) and to UCMS-CBD (p<0.01) rats (n=8-10 in each group).

Experimental Design:



To explore the neuroprotective effects of CBD on AD onset and progression in STZ model rats, CBD will be injected for two weeks after STZ or at defined time points (after STZ, at 3 and 6 months). Adult (2-month-old) and middle-aged (15-month-old) male and female rats will be randomly divided into 4 groups: aCSF+Vehicle, aCSF+CBD, STZ+Vehicle, and STZ+CBD. Rats will then receive an ICV injection of STZ or artificial cerebrospinal fluid (aCSF; Day 0) in the left ventricle. One day after the stereotaxic procedure (Day 1), all rats will begin receiving daily i.p. injections of CBD (10 mg/kg) or vehicle for 14 consecutive days. Tail blood will be collected on Days 1 and 14. Behavioral tests will be performed from Day 15 to Day 29 in the following order: open field (OF; Day 15), object location [OL; Day 19, after 3 days of habituation (HABIT) to the arena], novel object recognition (NOR; Day 20), episodic-like memory (ELM, Day 21), social preference and social recognition (Day 22), elevated plus maze (EPM, Day 23), Morris water maze (MWM, Days 24-28). At the end of the experiment (Day 30), brains and blood will be collected for biochemical analyses. In a second study, adult (2-month-old) male and female rates will receive an ICV injection of STZ or aCSF in the left ventricle (Day 0). Then, rats will treated with CBD (10 mg/kg) or Vehicle for 14 days, followed by behavioral and cognitive evaluations as described above. These treatment and behavioral assessments will be performed three times during the experiment at the following time points: one day after the stereotaxic procedure (day 1), 3 months later, and 6 months thereafter. At the end of the experiment, tissues will be collected from these animals for biochemical analyses.

**Aim 2:** **To determine whether the HPC-PFC pathway plays a fundamental role in shaping the abnormal cognitive and emotional behaviors associated with AD.**

In thiswe ask whether HPC input to PFC is central in controlling the behavioral phenotype in AD rats; to that end we will use a chemogenetic dual-virus approach (Boender et al 2014) to selectively inhibit the glutamatergic projections from HPC to the PFC and examine whether reducing glutamate release can induce cognitive decline and emotional dysfunction as observed in our STZ rats. This would suggest that the HPC-PFC pathway plays a fundamental role in abnormal cognitive and emotional behaviors associated with AD.

The monosynaptic unidirectional projection of neurons extending from the ventral subiculum of the HPC to the medial PFC [mPFC, infralimbic (IL), prelimbic (PL)] is critically involved in executive functions and emotional regulation (Godsil et al., 2013). Hence, disruptions in HPC-PFC functional connectivity induced using Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based chemogenetic tools can contribute to cognitive decline and neuropsychiatric symptoms such as depression and anxiety. Chemogenetic technologies are frequently used to investigate the neural mechanisms of behavior (Smith et al., 2016), and are ideally suited to studying the role played by HPC-PFC excitatory transmission for several reasons: (i) these tools allow for cell-type specificity in distinct anatomical brain regions, through the use of viral constructs carrying a cell-type-specific promoter (e.g., CamKII); (ii) they enable relatively long-term reductions in activity [up to 6 h (Zhu et al., 2014)], which are particularly well-suited to conducting a series of behavioral studies; and (iii) rather than complete excitation or inhibition of all cells in a desired population, DREADD-induced changes lead to more physiologically meaningful changes in the patterns of activity, since their effects are mediated by endogenous intracellular signaling mechanisms (Armbruster et al., 2007). Our preliminary studies, collaborating with Prof. Inna Gaisler-Salomon in our department, have confirmed our ability to direct a DREADD-carrying virus to a specific cell population wherein it is expressed following viral delivery (Figure 4).

10x magnification of fluorescence images following the injection of ssAAV-8/2-mCaMKIIa-hM4D(Gi)mCherry-WPRE-hGHp(A) (0.3 µL) into the hippocampus. Brains were perfused 21 days post-injection. 35 micron slices were cut and stained with 0.5% DAPI.



Experimental Design:



A Cre-dependent AAV2 vector (AAV2-hSyn-DIO-hm3D-mcherry, 5.5 × 1012 vg/ml; 0.5 μl/side at 0.1 μl/min) will be injected bilaterally into the ventral subiculum. The retrograde-traveling CAV2-Cre virus (1–1.2 × 1012 vg/ml; 0.75 μl/side at 0.15 μl/min) or a control mCherry virus (1–1.2 × 1012 vg/ml; 0.75 μl/side at 0.15 μl/min) will be microinjected into the IL or PL (in different groups) (Boender et al., 2014; Kerstetter et al., 2016; Marchant et al., 2016). Rats will be allowed to recover for 4 weeks before STZ to allow for robust gene expression in target regions. The DREADD agonist clozapine N-oxide (CNO; dissolved in drinking water at a concentration of 0.25 mg/ml; Carvalho Poyraz et al 2016) will be chronically administered to rats in drinking water for 2 weeks after which behavioral testing will be performed without CNO )from Day 15 to Day 28, as described in Experiment 1(. Additionally, sham/CNO controls will be included since the CNO metabolite clozapine may affect physiology and behavior by binding to receptors other than DREADDs (Gomez et al 2017; MacLaren et al 2016). The experiment will thus consist of the following groups in aCSF and STZ injected rats: hM3D (HPC) –Cre (IL); hM3D (HPC) –Veh (IL); hM3D (HPC) –Cre (PL) and hM3D (HPC) –Veh (IL). Rats will be decapitated at the end of behavioral testing. We will use immunohistochemistry to verify virus expression. We hypothesize that glutamatergic inhibition in these pathways may induce AD-like alterations in cognitive and emotional function, suggesting that the HPC-PFC pathway is crucial for these effects.

**Aim 3: To explore whether inhibiting or activating specific candidate miRNAs in the HPC-PFC can reverse AD-related cognitive and emotional dysfunction, and to determine whether CBD can protect against AD phenotypes through miRNA-mediated mechanisms.** In our final Aim, we will explore whether different miRNAs are critically involved in AD-related cognitive and emotional dysfunction and the therapeutic effects of CBD by using agomirs and antagomirs to activate or inhibit specific miRNAs in the HPC-PFC pathway, after which the association between changes in miRNA expression, cognitive/emotional pathology, inflammatory markers, CBD targets, AD pathology-related targets, and β-catenin will be assessed. miRNAs are crucial regulators of gene expression and promising candidates for biomarker development. As such, the activation or silencing of particular miRNAs may be ideally suited to treating cognitive disorders and dementia (Bahlakeh et al., 2021). To determine whether specific miRNAs can have therapeutic-like effects in STZ rats and to determine whether the therapeutic-like effects of CBD are mediated by specific miRNAs, we will use a viral approach to inhibit/activate specific miRNAs in the HPC-PFC pathway. We have preliminary findings that microinjecting antagomir-16 (anti-mir, 20 nm) into the right ventricle significantly decreases the expression of miR-16 in the PFC (Figure 5). Based on our findings from Aim 1, specific miRNAs will be activated or inhibited in an attempt to ameliorate STZ-induced alterations in cognitive and emotional function. In another set of rats, a specific miRNA will be activated or inhibited to exacerbate STZ-induced alterations in memory and emotional function or to block therapeutic-like effects of CBD in STZ males and females.

Rats were microinjected with antagomir-16 (anti-mir, 20 nm) into the mPFC and decapitated after one week. Left:  histological verification of the site of microinjection. Right: A significant decrease was observed in the expression of mir-16 in the PFC (\*, p<0.05).



Experimental Design

Adult (2-month-old) and middle-aged (15-month-old) male and female rats will receive ICV injection of STZ or aCSF to the left ventricle and an agomir or antagomir to the right ventricle (Day 0), after which the study will be completed as in Aim 1. Rats will be decapitated on Day 30, and the association between changes in miRNA expression, cognitive/emotional pathology, inflammatory markers, CBD targets, AD pathology-related targets, and β-catenin will be assessed. If we find that silencing/activating miRNAs does not ameliorate STZ-induced dysfunctional cognitive and emotional function, we will increase Wnt/β-catenin pathway by blocking the phosphorylation of GSK-3β or overexpressing β-catenin in the HPC-PFC pathway.

 **Methods:**

**Subjects:** Adult (60 days old) and middle-aged (15 months)male and female Sprague-Dawley rats will be group housed at 22 ± 2°C under 12-hour light/dark cycles with *ad libitum* food and water access. Appropriate measures will be taken to minimize the number of animals used and their suffering.

**STZ model for sporadic AD:** Rats will be anesthetized with a mixture of ketamine/xylazine and placed in a stereotaxic frame (Stoelting). STZ (3 mg, 10μl) or aCSF will be ICV injected to the left ventricle (AP: −0.8 mm, ML: +1.5 mm, DV: −3.6 mm from dura). **Agomir and Antagomir** constructs will be ICV injected into the right ventricle (20 nmol). For **chemogenetic inhibition,** viruses will be delivered to the ventral subiculum. The injection volume and flow rate will be controlled using a micromanipulator (2 µL/min; Viral Vector Facility, ETH Zurich), after which the syringe needle will remain in place for an additional 10 min to prevent reflux.

**Behavioral battery** (Abush and Akirav, 2012; Bauminger et al., 2022;Burstein et al., 2018):The **OF** test assesses general locomotor function (total distance, cm, divided into 5 min bins) and novelty-induced anxiogenic behavior (time in arena center, first 5 min). The **NOR** test, with an inter-trial interval (ITI) of 5 min, is used to measure novelty recognition and working memory. The **OL** version of this test assesses spatial memory. We will assess total exploration time (s) and the mean discrimination-index (DI), calculated as TN/TN+TF (TN = novel object exploration time, TF = familiar object exploration time) in the test phase. In the **ELM**, animals spontaneously explore an environment and attempt to associate an object (What), its location (Where), and the temporal context (first or second occurrence – When). The task consists of two sample trials and a test session of object exploration of 5 min each. 1h intervenes between each trial. Compared to samples, objects could be stationary (A1 and B1) or in different locations (A2 and B2). Animals tend to explore A1 > B1 (“temporal pattern”), B2 > B1 (“spatial pattern”) and A1 > A2 (“integrative pattern”). A1: old stationary; A2: old displaced; B1: recently stationary; B2: recently displaced (Chao et al., 2014). The **SP and SR** task includes two phases: initial familiarization with an unfamiliar juvenile rat and a novel object (which constitutes a social preference test), followed by a recognition phase, with the previously familiarized rat and a novel juvenile (which constitutes a social recognition test). The **EPM** is used to assess anxiety-related behavior; rats are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer simultaneously for 5 min. The **MWM** test is also a test of spatial learning that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform.

**Drugs:** CBD (10 mg/kg) will be freshly prepared and administered in 1 mL/kg of vehicle. This dose was selected based on previous work (Campos et al 2013) and our preliminary results.

**Real-time (RT) PCR and western blots (WB):** Brain tissues are collected to detect miRNAs expression. A 50–100 mg tissue sample is mixed with 1ml Trizol (Invitrogen) to extract total RNA from frozen samples. One microliter of RNA is used to measure the expression of miRNAs using RT-PCR. The expression of U6 is used as internal control. The expression of miRNAs is calculated according to the threshold cycle (CT). The CT of the target gene for each sample is corrected by subtracting the CT of the internal control (ΔCT). The controls are chosen as reference samples with mean ΔCT for the control samples being subtracted from the ΔCT for all experimental samples (ΔΔCT). Finally, the relative expression levels are calculated as 2−[(CT of a specific miRNA) − (CT of U6)].

Rats will be euthanized and brain tissues from the IL, PL, ventral subiculm and dorsal hippocampus will be harvested, with whole blood additionally be collected for biochemical analysis. RNA extraction, cDNA preparation, and qRT-PCR will be performed as previously described (Bauminger et al., 2022) to detect the expression of miRNAs and mRNA. {rotein levels will be determined via Western blotting as previously described (Alteba et al., 2021). miRNAs: miR-9, miR-29abc, miR-34a, miR-107, miR-125b, miR-132, miR-146a, miR-155Mrna; **CBD main targets**: cannabinoid receptors 1 and 2 (*CNR1* and *CNR2*, respectively), FAAH, the enzyme that hydrolyzes the endocannabinoid anandamide, and the serotonergic receptor 5HT1A (*htr1a*). **Proteins markers associated with AD pathology**: Aβ protein, phosphorylated Tau (p-Tau Ser396) protein; **β-catenin** (1:5,000; abcam, UK; ab32572 [E247]). We will also investigate **peripheral miRNAs as potential biomarkers** of AD and treatment response and **pro-inflammatory cytokines** (IL-1β, IL-6, and TNF-α) in whole blood. For Western blotting samples will be resolved via SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Blots are incubated with the specific primary antibodies (abcam) overnight at 4°C, followed by washing and 1h incubation with an HRP-linked secondary antibody. Protein samples are standardized with β-actin (cell signaling; 1:1,000).

**Estrus cycle and estrogen (females):** The estrous cycle will be examined daily in the morning by collecting vaginal cytology samples as previously described (Zer-Aviv and Akirav 2016). Levels of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α in whole blood will be evaluated via sandwich ELISA according to the manufacturer’s instructions (Abcam and R&D systems).