Received: 3 December 2024 | Revised: 11 February 2025 | Accepted: 13 February 2025

DOI: 10.1002/alz.70081

Alzheimer's & Dementia®
THE JOURNAL OF THE ALZHEIMER'S ASSOCIATION

RESEARCH ARTICLE

Commonly prescribed multi-medication therapies exert sex-specific effects on Alzheimer's disease pathology and metabolomic profiles in *App*$^{NL\text{-}G\text{-}F}$ mice: Implications for personalized therapeutics in aging

Francesca Eroli[1] | **Kristina Johnell**[2] | **Zeynep Acararicin**[1] | **Christina Tsagkogianni**[1] | **Stefania Zerial**[1] | **Saverio Lancia**[1] | **Maria Latorre-Leal**[1] | **Vilma Alanko**[1,3] | **Sarah N. Hilmer**[4] | **Anna Matton**[1,3] | **Jonas W. Wastesson**[2] | **Angel Cedazo-Minguez**[1] | **Silvia Maioli**[1]

[1]Department of Neurobiology, Care Sciences and Society, Center for Alzheimer Research, Division of Neurogeriatrics, Karolinska Institutet, Solna, Sweden

[2]Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Solna, Sweden

[3]Department of Neurobiology, Care Sciences and Society, Center for Alzheimer Research, Division of Clinical Geriatrics, Karolinska Institutet, Solna, Sweden

[4]Kolling Institute, Northern Sydney Local Health District and The University of Sydney, St Leonards NSW, Australia

Correspondence
Silvia Maioli and Francesca Eroli, Department of Neurobiology, Care Sciences and Society, Center for Alzheimer Research, Division of Neurogeriatrics, Karolinska Institutet, Visionsgatan 4, 17164, Solna, Sweden.
Email: silvia.maioli@ki.se and francesca.eroli@ki.se

Funding information
Margaretha af Ugglas Foundation; King Gustaf V:s and Queen Victoria Foundation; The private initiative "Innovative ways to fight Alzheimer's disease -Leif Lundblad Family and

Abstract

INTRODUCTION: Polypharmacy is common among older adults and people with dementia. Multi-medication therapy poses risks of harm but also targets comorbidities and risk factors associated with dementia, offering therapeutic potential.

METHODS: We evaluated the effects of two polypharmacy regimens and monotherapies on male and female *App*$^{NL\text{-}G\text{-}F}$ knock-in mice. We assessed functional, emotional, and cognitive outcomes;amyloid pathology; and serum metabolomics profiles.

RESULTS: A combination of metoprolol, simvastatin, aspirin, paracetamol, and citalopram improved memory, reduced amyloid burden and neuroinflammation, and modulated AD-associated metabolomic signatures in male mice, with negligible effects in female mice. Substituting two cardiovascular drugs impacted emotional domains but worsened memory, predominantly in female mice. In males, monotherapies could not explain the combination effects, suggesting drug synergy, whereas in female mice, certain monotherapy effects were lost when combined.

DISCUSSION: This study uncovers the sex-specific effects of polypharmacy in an AD model, identifying mechanisms and biomarkers that can guide gender-specific use of medicines in dementia prevention and management.

KEYWORDS
aging, Alzheimer's disease, amyloid plaques, antidepressant, behavior, cardiovascular drugs, combination therapies, dementia, memory, metabolomics, microglia, mouse models, polypharmacy, sex differences, statins

others"; Swedish Research Council; The regional agreement on medical training and clinical research ALF; Alzheimerfonden, Grant/Award Number: AF-1011030; Stiftelsen för Gamla Tjänarinnor; Gun och Bertil Stohnes Stiftelse; Olle Engkvists Stiftelse, Grant/Award Number: 231-0067

Highlights

- Two polypharmacy combinations show sex-specific effects on AD pathology and serum metabolomic profiles.
- Metoprolol+simvastatin+aspirin+paracetamol+citalopram improves memory and amyloid pathology in male mice.
- Replacing metoprolol and simvastatin with enalapril and atorvastatin eliminates benefits in male mice and impairs memory in female mice.
- Selected monotherapies produce sex-specific effects but only partially explain the outcomes of the combinations.
- Metabolomic pathways in serum indicate possible mechanisms and biomarkers for evaluating the effectiveness and safety of personalized therapies in aging and dementia.

1 | BACKGROUND

Alzheimer's disease (AD) and -related dementias (ADRD) are a group of neurodegenerative disorders, with AD accounting for 60%–80% of dementia cases.[1] Aging is the main risk factor, and with the population 65 years of age and older growing, dementia cases could reach 150 million by 2050,[2] posing a substantial health care challenge. Although the recent approvals of anti-amyloid compounds (aducanumab, lecanemab, and donanemab) are promising for disease-modifying treatments,[3] these medications are still under evaluation and might be suitable only for specific patient populations. AD is a highly heterogeneous disorder with variation in pathology onset and progression among individuals.[4] The presence of comorbidities in people with ADRD further contributes to clinical phenotype diversity, increasing the challenge of finding effective therapies.[5,6] Several of these conditions are also recognized as risk factors for dementia, including diabetes, depression, hypercholesterolemia, and cardiovascular diseases.[3] The 2024 Lancet Commission report suggested that 45% of dementia cases could be prevented by addressing lifestyle risk factors,[3] indicating that targeting modifiable factors may reduce ADRD incidence. Due to the prevalence of the abovementioned multiple conditions in aging and ADRD, older adults and people with dementia are frequent users of multi-medication therapies (i.e., polypharmacy, the use of five or more drugs simultaneously).[7,8] Drug classes that are among the top prescribed in multidrug therapies (cardiovascular and psychotropic medications)[9,10] also target some of the conditions identified as risk factors for dementia. In the current pipeline of AD drug development, 31% of the candidate therapies are repurposed agents,[11] where antihypertensives, antidiabetics, and psychotropic compounds are assessed for their potential to prevent or slow AD pathology. Thus, multi-medication therapies commonly used in the older population may exert a protective role by addressing comorbidities linked to ADRD.[12] In alignment with this, a meta-analysis[13] found that certain drug combinations correlate with a reduced rate of cognitive decline. On the other hand, the long-term use of multiple-drug regimens is also associated with negative effects on physical function in older adults, like reduced muscle strength and increased frailty.[14,15] Epidemiological studies report that polypharmacy heightens the risk of cognitive decline[16] or exacerbates neurodegenerative processes in mild cognitive impairment (MCI) patients and dementia patients.[17] Clinical interventions found that de-prescribing was associated with reduced cognitive decline.[18–21] Nevertheless, there is no experimental evidence on the effects of polypharmacy on AD onset and progression, and preclinical studies in rodents were performed only in wild-type mice, showing that polypharmacy impacts functional and cognitive outcomes with age- and sex-dependent effects,[22–25] and affects the microbiome and hepatic proteome.[26]

Biological sex- and gender-related factors differentially impact pharmacology, polypharmacy, and neurodegenerative disorders.[27] Men and women with ADRD exhibit differing incidence rates and disease progression[28] due to biological and gender-related factors, such as genetic background, immune responses, sex hormones, and lifestyle.[28,29] Some of these aspects differentially impact drug metabolism and efficacy in men and women.[27] Furthermore, the prevalence of polypharmacy is higher among older women than among men.[8] About 40% of preclinical pharmacological studies used male mice,[30] and when both sexes were used, 73% of these studies reported sex-specific outcomes on health/life span, supporting the need for sex-stratified research. Taking all these aspects into consideration, a careful evaluation of the benefits and drawbacks of multi-medication use is vital to optimize chronic condition management and ensure tailored treatment strategies for specific patient categories.

This study aims to address the knowledge gap regarding the effects of multi-medication therapies on ADRD-related pathology and the underlying mechanisms. We selected two polypharmacy combinations commonly prescribed in older adults based on our previous population-based study[10] and investigated their effects in early-stage AD in male and female $App^{NL\text{-}G\text{-}F}$ knock-in mice. We tested selected monotherapies to compare their effects with those of the multi-medication therapies. To our knowledge, this is the first preclinical study examining whether polypharmacy can affect behavioral domains, amyloid pathology, and metabolomic profiles in a transgenic

mouse model. Using a back-translational approach, our findings offer new insights into potential sex-specific differences, molecular mechanisms, and serum biomarkers of polypharmacy effects in the context of AD pathology. These experimental results can serve as a foundation for designing and optimizing more effective and gender-tailored therapeutic strategies, ultimately enhancing personalized approaches to AD management.

RESEARCH IN CONTEXT

1. **Systematic review**: We used PubMed to review relevant literature on polypharmacy use in aging and Alzheimer's disease and related dementias (ADRD). Analgesics, antithrombotics, lipid-modifying agents, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, and antidepressants are top-prescribed drug classes in polypharmacy. Epidemiology shows both the risks and benefits of these drugs and their combinations on ADRD, but preclinical evidence on the effects of polypharmacy on disease pathology is lacking.
2. **Interpretation**: Polypharmacy exerted sex-specific effects on behavior, amyloid beta (Aβ), neuroinflammation, and serum metabolomics profiles in mice. A drug combination rescued AD-related pathological features in male mice but showed no effect in female mice. Substitution of two cardiovascular drugs eliminated these benefits in male mice and impaired memory in female mice. Monotherapies displayed sex-specific outcomes that were either enhanced or nullified when combined.
3. **Future directions**: Research should examine sex-specific mechanisms and identify biomarkers for polypharmacy events. Translating findings with sex-stratified clinical studies and testing alternative regimens will help optimize gender-tailored therapies in aging and ADRD while minimizing risks.

2 | MATERIALS AND METHODS

2.1 | Animals

We used male and female homozygous *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in (APP KI) transgenic mice and age-matched wild-type (WT) mice of the same background (C57BL/6J) as controls. The APP KI mouse model recapitulates key features associated with AD pathology, displaying a rapid progression of amyloid pathology due to the expression of three familial AD mutations (Swedish, Iberian, and Arctic), which lead to increased pathogenic amyloid beta (Aβ) levels and enhanced Aβ aggregation starting at 2 months of age.[31] The first signs of a slight cognitive impairment appear in Y Maze and fear conditioning (FC) tests at 6 months and worsen by 12 months of age.[32] Mice were housed under controlled conditions (21 ± 1°C temperature, 55 ± 5% humidity, and 12 h light/dark cycle) in cages of four to five animals, with food and water provided ad libitum.

All animal experiments were performed in accordance with the local national animal care and Swedish guidelines and approved by the Swedish Board of Agriculture (ethical permit ID 827/2017 and ID 11607-2022). Every possible effort was made to minimize animal suffering and distress.

2.2 | Experimental design

As shown in the experimental design in Figure 1, we divided the animals into three sex-stratified cohorts and randomly assigned the APP KI mice to a control or a drug treatment, whereas WT mice were on the control diet. Before the start of the study, all animals were acclimatized to the standard chow for 10 days (control diet, 18.5% protein, 5.5% oil and fat, 4.5% fiber; Teklad 2918 diet, Research Diet Inc., NJ, USA[22]). We initiated the drug treatments at 4.5 months of age, prior to cognitive deficits but already in the presence of some AD pathological features, for a total duration of 8 weeks. Behavioral testing was performed over the last 4 weeks of treatment. At the end of the study period, we euthanized the animals and collected trunk blood and brain tissues.

We monitored the health status of the mice and their diet intake by measuring body weight (BW) and food intake (FI; g food/mouse/day) weekly. Creatinine and alanine transaminase (ALT) levels were analyzed in the serum, according to the kit protocols (DICT-500, BioAssay Systems, and MAK052, Sigma-Aldrich) as described previously by our group.[22]

2.3 | Drug treatments

The selection of drugs included in the multi-therapy combinations of this study was based on a population study of individuals ≥75 years of age in Sweden, which assessed the most commonly prescribed drug classes and compounds used in polypharmacy.[10] Mice from Cohort 1 were administered a control or a multi-medication diet (Combination 1), which we previously tested in WT mice in our lab.[22,23] Combination 1 comprises the following drugs: metoprolol (100 mg/kg/day; Sigma-Aldrich, USA), paracetamol (acetaminophen, 100 mg/kg/day; Sigma-Aldrich, USA), aspirin (acetylsalicylic acid, 20 mg/kg/day; Sigma-Aldrich, USA), simvastatin (10 mg/kg/day; Selleck Chemicals, USA), and citalopram (10 mg/kg/day; Selleck Chemicals, USA), which belong to the classes of beta-blockers, analgesics, antithrombotic, lipid-modifying, and antidepressant agents, respectively. Among cardiovascular drug classes, beta-blockers are the most used, followed by diuretics and angiotensin-converting enzyme (ACE) inhibitors.[10] Mice from Cohort 2 were fed with a control or a second multi-medication regimen (Combination 2) where we replaced two compounds from Combination 1: the beta-blocker (metoprolol) with an ACE inhibitor, enalapril (10 mg/kg/day; Cayman Chemicals, USA), and simvastatin with atorvastatin (15 mg/kg/day; Cayman Chemicals, USA). Both the fungal

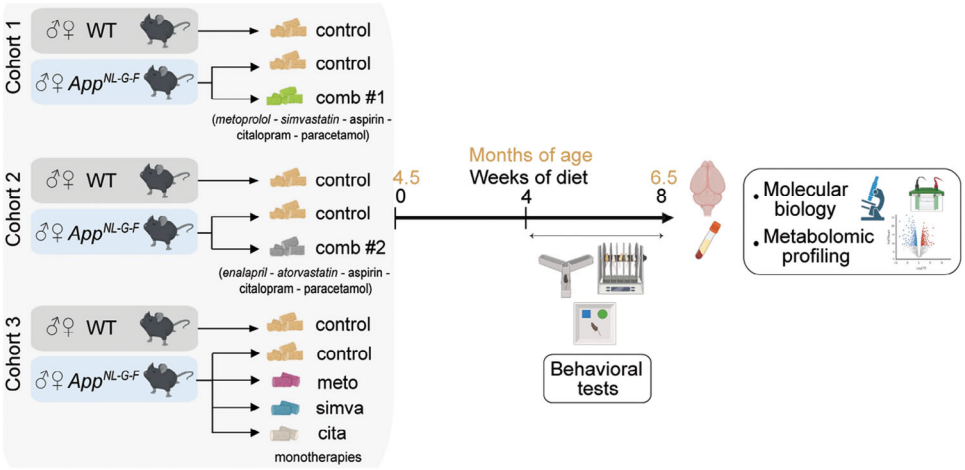


FIGURE 1 Experimental design. Male and female WT and APP KI mice from three different cohorts were administered either a control or a drug regimen, starting at the age of 4.5 months over a total of 8 weeks. Behavioral assessment and tissue collection were performed as indicated by the timeline. Comb 1 = Combination 1 (metoprolol, simvastatin, aspirin, citalopram, paracetamol); Comb 2, Combination 2 (enalapril, atorvastatin, aspirin, citalopram, paracetamol); meto, metoprolol; simva, simvastatin; cita, citalopram. Created with BioRender.com. App^{NL-G-F}, $App^{NL-G-F/NL-G-F}$ knock-in; WT, wild type.

simvastatin and synthetic atorvastatin are highly prescribed lipid-modifying agents in Sweden,[10,33] with different effects on cognitive decline possibly depending on their different biochemical properties.[34] Mice from Cohort 3 were administered a control diet or a monotherapy regimen from three selected drugs used in combination at the same dosage: metoprolol, simvastatin, or citalopram.

As described in detail in our previous studies with multi-medications in mice,[22,23] compound dosages per kg/BW were derived from the human therapeutic range and adjusted for mice, considering interspecies differences in pharmacodynamics and pharmacokinetics. Final drug dosages were determined from previous studies in rodents, where the same drugs were administered at similar doses with efficacy and without exhibiting toxicity. To account for potential variations between the predicted and actual FI of the animals and ensure adequate exposure to the compounds, concentrations were at the higher end of the previously established therapeutic dosing ranges (see Table S1). Medication amounts added to the diets were calculated considering a mean FI of 0.1 ± 0.2 g food per gram of mouse per day, as reported before in our lab and in the literature.[35]

2.4 | Behavioral testing

We evaluated the animals across various behavioral domains, focusing on functional, emotional, and cognitive outcomes through a diverse array of behavioral tests as follows: open field (OF), Rotarod, Grip Strength, Elevated Plus Maze (EPM), Y Maze, novel object recognition (NOR), forced swim test (FST), and FC. The procedures were conducted in the order listed, starting from the least stressful tests, and allowing a break for a few days for the mice to recover between the more demanding tasks. The experiments were run in white light between 8:00 and 16:00 by the same female experimenter. OF, Rotarod, Grip Strength, EPM, Y Maze, NOR, and FC tests were performed and analyzed as described previously.[22,23] Mice that performed less than nine arm entries in the Y Maze test, and that explored the objects for less than 15 s during Day 2 of the NOR test were not included in the analysis. In FST, the mice were placed in a clear acrylic glass cylinder filled with water (20 cm diameter x 30 cm height; 23 ± 1°C water temperature) for a 6 min session recorded with a camera. The immobility % was analyzed in the last 4 min of the test.[36]

Data from OF locomotor arenas were acquired through TSE Acti-Mot software (TSE Systems GmbH, Germany), whereas the other test data were collected using the video-tracking system Ethovision XT-17 (Noldus, Netherlands), through a camera located above the apparatus, or in front, in the case of FST.

2.5 | Immunofluorescence staining

We performed immunohistochemistry (IHC) staining in APP KI brains. Fixed half-brains were embedded in paraffin and then sliced into 5-μm-thick coronal slices at the ZeMac Histoanalysis facility (Karolinska Institute, Huddinge, Sweden). After deparaffinization, antigen retrieval was done by incubating the sections with 1X Diva Decloaker (Biocare Medical) for 10 min at 110°C. Slices were permeabilized in 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 15 min. After washing, we blocked them in a solution of 5% goat serum in PBS (0.05% Tween [PBS-T]) for 30 min, followed by primary antibodies incubation at 4°C overnight. We used the following primary antibodies: mouse anti-Aβ 82E1 (1:1000; #10323, IBL, Japan) and rabbit anti-Ionized calcium-binding adapter molecule1 (Iba1, 1:1000; #019-19741, Wako, Japan). After three washes with PBS-T, we incubated the sections for 2 h with the following secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG (1:200; Invitrogen) and Alexa Fluor 546 goat anti-rabbit IgG (1:500; Invitrogen). After three washes, we did a 15 min incubation with 1 mg/mL 4′,6-diamidino-2-phenylindole (DAPI; 1:1000),

rinsed the slices again, and then mounted them with Gold Antifade Mountant mounting medium (Invitrogen). We acquired immunofluorescence images of whole half-brain slices with a 20X objective in the AxioScan Z1 fluorescence microscope (Zeiss, Germany). Aβ plaque load was quantified in two areas (region of interest [ROI]) in the cortex. Amyloid plaque number and area were quantified using the same ROIs in each brain section (two sections/mouse brain for a total of three to four mice per group) in ImageJ software (version 1.54f, National Institutes of Health [NIH], Bethesda, Maryland, USA) after thresholding the positive fluorescence signal.

2.6 | Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Cerebral Aβ deposits were quantified upon extraction of soluble and insoluble protein fractions[31]. We homogenized cortical samples from APP KI mice in Tris buffer (50 mM Tris-HCl, 150 mM NaCl buffer, pH 7.6, containing protease/phosphatase inhibitor cocktail). We centrifuged at 70,000 rpm for 20 min at 4°C to obtain the Tris-soluble (TS or soluble fraction) fraction as supernatant. Guanidine-HCl was added to TS fraction to give a 0.5 final concentration prior to enzyme-linked immunosorbent assay (ELISA). We sonicated the remaining pellet in 6 M guanidine-HCl buffer (containing protease/phosphatase inhibitor cocktail) and centrifuged at 70,000 rpm for 20 min at 4°C. After that, the supernatant was collected as a guanidine-soluble (GS or insoluble fraction) fraction and diluted to reduce the concentration of guanidine-HCl to 0.5 M. We determined the levels of Aβ40 and Aβ42 in both fractions by using ELISA kits #27713 and #27711 (IBL, Japan) according to kit instructions.

2.7 | Western blotting

We ran western blot experiments on cortical TS fraction lysates as described previously.[22,23] Nitrocellulose membranes were incubated overnight at 4°C with the following primary antibodies: anti-APP (1:1000, #MAB348, Sigma-Aldrich), anti-Iba1 (1:1000, #NBP2-16908, Novus), anti-Glial Fibrillary Acidic Protein (GFAP, 1:1000, #ab7260, Abcam), and anti-α-tubulin (1:10,000, # T9026, Sigma-Aldrich). Incubations with secondary antibodies were performed at room temperature for 2 h with anti-rabbit or anti-mouse IgG at 1:10,000 dilutions (LI-COR Biosciences GmbH, Germany). Immunoreactivity was measured by infrared fluorescence on LI-COR Odyssey system (LI-COR Biosciences, USA) and quantified with ImageJ software (version 1.54f, NIH, MA, USA).

2.8 | RNA extraction and real-time PCR (RT-qPCR)

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR) was carried out as described before.[37] We extracted total RNA from mouse hippocampi using RNeasy Mini Kit (#74106, Qiagen), followed by retro transcription with high-capacity cDNA Reverse Transcription Kit (#4368814, Applied Biosystems). We performed RT-qPCR amplification of genes of interest using Taqman Universal Master Mix (Applied Biosystems) and the following primers: *App*, Beta-Site APP-Cleaving Enzyme 1 *(Bace1)*, Presenilin 1 *(Psen1)*, A Disintegrin And Metalloproteinase Domain-Containing Protein 10 *(Adam10)*, Transmembrane Protein 119 *(Tmem119)*, Allograft Inflammatory Factor 1 *(Aif1)*, Purinergic Receptor P2Y, G-Protein Coupled, 12 *(P2ry12)*, Triggering Receptor Expressed on Myeloid Cells 2 *(Trem2)*, C-Type Lectin Domain Family 7a *(Clec7a)*, Cluster of Differentiation 68 *(Cd68)*, Interferon-Induced Protein with Tetratricopeptide Repeats 3 *(Ifit3)*, Interferon Regulatory Factor 7 *(Irf7)*, and Glyceraldehyde-3-Phosphate Dehydrogenase (*Gapdh)* (Applied Biosystems). All target genes were quantified relatively to *Gapdh* mRNA levels as endogenous control.

2.9 | Targeted metabolomics

2.9.1 | Metabolite extraction and LC/GC-MS

Metabolite extraction, identification, quantification, and primary quality control were carried out at the Swedish Metabolomics Centre (Umeå, Sweden), on serum samples (25 µL/sample) from WT and APP KI mice. Detailed information on the methodology can be found in Supplementary Material. By targeted processing approach 309 metabolites were identified by liquid chromatography/gas chromatography-mass spectrometry (LC/GC-MS), and raw data of metabolite readings were provided for subsequent analysis.

2.9.2 | Metabolomic analysis

Data analysis was conducted using MetaboAnalyst 6.0 software.[38] Data were auto-scaled (mean-centered and divided by the standard deviation [SD] of each variable) and processed with a statistical analysis module (one factor) for principal component analysis (PCA) and the analysis of differentially expressed metabolites. Metabolite set enrichment analysis (MSEA) was run based on the list of differential metabolites, identified by HMDB ID, and using the small molecule pathway database (SMPDB) library.[39]

2.10 | Statistical analysis

For the analysis of behavioral tests, western blotting, IHC, ELISA, and RT-qPCR, data are presented as mean ± standard error of the mean (SEM), with *n* indicating the number of animals. GraphPad Prism 10 (San Diego, CA, USA) was used to perform the statistical analyses. When comparing two groups, we used Student's *t*-test or the nonparametric Mann–Whitney test. One-way or repeated-measures two-way analysis of variance (ANOVA) was applied to analyze datasets with one or two independent variables, respectively. Tukey's test was used to

control for multiple comparisons in post hoc analyses with adjusted *p* values (reported in figure captions) for significant comparisons. *F* and *p* values for the ANOVA were provided (in figure captions) along with information on whether the effect of time or group was examined. Statistical significance was set at $p \leq 0.05$.

For metabolomic analysis, the threshold for significantly differential metabolites was set to fold change (FC) ± 1.5 (FC >1.5 for upregulated and <0.67 for downregulated metabolites) and *p* value < 0.05.[40] For MSEA, enriched pathways were considered statistically significant with $p \leq 0.05$ (Over Representation Analysis-ORA, MetaboAnalyst 6.0).

3 | RESULTS

3.1 | Multi-medication therapies do not impact health parameters in male and female APP KI mice, and citalopram monotherapy leads to weight gain only in male mice

Three cohorts of 4.5-month-old male and female WT and APP KI mice were administered control or drug diets for 8 weeks, as illustrated in the experimental design in Figure 1. No sign of illness or mortality was observed over the study period, indicating that the animals tolerated the multiple-drug and monotherapy treatments well. We registered a general increase in BW in most groups over time, as typically observed at this age. APP KI male mice administered Combinations 1 and 2, as well as their WT and APP KI control groups, significantly gained weight over the 8 weeks (Figure S1A and SB, for Cohorts 1 and 2, respectively). A similar increase in BW was also observed in WT and APP KI males fed with control or monotherapy diets (Figure S1C), which was particularly evident in the citalopram group. The food consumption during the 8 weeks of treatment was stable in male mice, with small variations over the weeks (Figure S1D–F). APP KI female groups fed with control or drug regimens also showed a gradual increase in their BW over time (Figure S1G–I, for Cohorts 1–3, respectively). Although both WT female groups from Cohorts 2 and 3 gained weight, WT female mice from Cohort 1 did not grow significantly over the 8 weeks (Figure S1G). Some variability in FI was detected for WT and APP KI female groups treated with control or medication regimens during the study period (Figure S1J–L, for Cohorts 1–3, respectively), especially in female mice administered monotherapy diets. In this cohort, we observed higher average FI during the first weeks compared to the other female cohorts, mainly in the metoprolol and simvastatin APP KI groups (Figure S1L). It is important to note that in some animal cages from Cohort 3, a significant amount of food pellets was often found crumbled in the cage bedding, suggesting that the food measured from the cage grid could not correspond to the one eaten by the mice. This might explain the higher average FI displayed by some groups. Overall, across all male and female cohorts, the measured FI was within the expected records according to our previous polypharmacy studies,[22,23] indicating that the final drug dose taken by the mice was within the expected range.

At the end of the treatment, we determined the levels of serum creatinine and ALT in the multi-medication cohorts of both sexes as indicators of renal and hepatic function, respectively. The levels of these markers did not differ in male and female APP KI mice treated with Combination 1 compared to control groups (Figure S1M). The Combination 2 diet did not affect the levels of ALT, whereas we observed a reduction of creatinine levels, however, still within healthy ranges, in APP KI male mice fed with this regimen relative to APP KI control mice (Figure S1N).

3.2 | Combination 1 lowers exploration, modulates anxiety-like behavior, and improves memory in APP KI male but not female mice

We first assessed the effects of the drug regimens on exploration and locomotion using OF and Rotarod tests. In OF locomotor arenas, APP KI male mice treated with Combination 1 traveled a shorter distance compared to APP KI controls (Figure 2A and Figure S2A, left plot), showed a lower average speed, and a higher percentage of time spent exploring the periphery than their control group (Figure 2B). In the Rotarod test, APP KI male mice outperformed WT mice by showing a significantly higher latency to fall from the rotor in Trial 3 and on average (Figure 2C and Figure S2D, left plot). This was observed consistently in all male cohorts (Figures 2C, 3C, and 4C). APP KI males fed with Combination 1 spent significantly less time on the rotor compared to APP KI controls, comparable to WT mice (Figure 2C and Figure S2D, left plot). Conversely, in female mice, there was no general effect of Combination 1, as no significant changes were found in OF distance moved (Figure 2D and Figure S2A, right plot) and speed (Figure 2E, left plot) for APP KI–treated mice relative to their APP KI control group, but only for the WT mice. No significant differences were observed between female groups in the Rotarod test (Figure 2F and Figure S2D, right plot).

We conducted the grip strength test to determine if the drug treatments affected muscle strength. Neither multi-drug combination affected this parameter in either sex (Figure S2G and H).

EPM test and FST were performed to evaluate anxiety- and depressive-like behaviors. APP KI mice have previously shown a greater willingness to explore open arms in EPM,[41] which we also found in our cohorts at this age, more pronounced in female mice (Figures 2, 3, and 4G and H). The Combination 1 diet reversed this behavior in APP KI male but not in female mice (Figure 2G and H, respectively). For FST, we did not find any genotype or treatment effect in either male or female mice from Cohort 1, as shown by a similar immobility % between groups (Figure 2I and J).

The APP KI mouse model is reported to show the first signs of memory and learning deficits in some behavioral tasks starting around 6 months.[42,43] In line with this, APP KI male mice did not alternate significantly above the 50% chance level in the Y Maze test (Figure 2K); conversely, the Combination 1–treated group showed a significantly better % of alternation in this test. In the NOR test, we did not observe an influence of the treatment on APP KI male mice (Figure 2L). Similarly

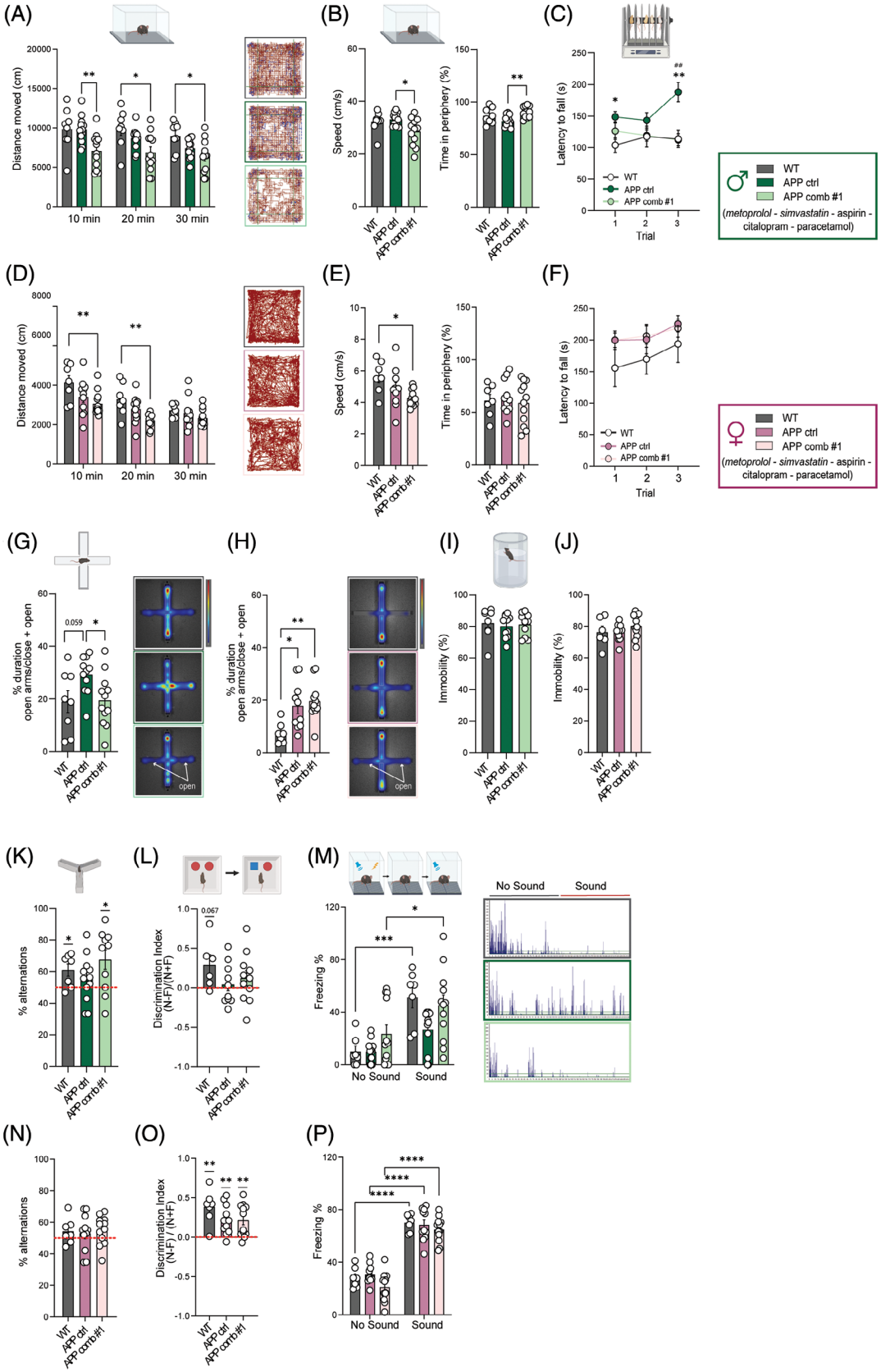


FIGURE 2 Effects of Combination 1 on behavioral outcomes in APP KI male and female mice. (A–C) OF test analysis in WT, APP ctrl and APP Comb 1 male mice from Cohort 1. (A) Distance moved measured over three 10 min time intervals. Repeated-measures two-way ANOVA, effect of group, $F = 6.26$, $p = 0.0055$, followed by Tukey's multiple comparison test: APP ctrl vs APP Comb 1 at 10 min interval $p = 0.00087$, WT vs APP Comb 1 at 20 and 30 min intervals, $p = 0.0390$ and $p = 0.0266$, respectively. To the right, representative pattern of movements of the total 30 min trial for WT, APP ctrl, or APP Comb 1 male mice. (B) Average locomotor speed (one-way ANOVA, $F = 4.13$, $p = 0.0264$, followed by Tukey's multiple

to the Y Maze test, this multiple-drug regimen exerted a positive effect on the FC cue task, as shown by a significant increase in the percentage of freezing after the sound cue in the APP KI mice administered Combination 1, conversely to APP KI control mice (Figure 2M). The FC context test (Figure S2I–K, left plot) did not unveil memory deficits in our cohorts of APP KI mice at this age, or an effect of the drug regimen. Unlike male mice, female mice fed with Combination 1 did not show any evident impact of the genotype or the treatment on the same cognitive tests, as shown in Figure 2N–P and Figure S2I, right plot.

3.3 | Combination 2 positively modulates anxiety-like behavior but worsens cognitive performance predominantly in female APP KI mice

We ran the same battery of behavioral tests in the cohorts fed with Combination 2 diet. APP KI mice from control or treated groups of both sexes reduced the distance moved only compared to WTs (Figure 3A and D, and Figure S2B), indicating that this polypharmacy regimen did not affect general locomotor activity in the APP KI mice. This treatment did not affect the average speed or time spent in the periphery of the OF arena in APP KI male mice (Figure 3B). In the Rotarod test, Combination 2 exerted in male mice an effect comparable to what was observed previously for Combination 1, significantly decreasing the latency to fall from the rotor in treated APP KI mice compared to controls (Figure 3C and Figure S2E, left plot). In female APP KI mice, the same drug regimen did not change the speed or the time spent in the OF arena's corners when compared to the APP KI control group (Figure 3E), or the performance on the Rotarod (Figure 3F and Figure S2E, right plot). In EPM and FST tasks, Combination 2 was more effective in APP KI female than male mice, as suggested by a significantly lower percentage of time spent in open arms and immobility time in the APP KI–treated group as compared to APP KI control mice (Figure 3G–J).

When evaluating cognitive tests, we found both WT and APP KI male groups to alternate above the chance level in the Y Maze test, although with a greater significance for WT mice (Figure 3K), whereas in the NOR test, APP KI male animals administered multi-medication diet 2 showed a lower discrimination index compared to WT and APP KI control groups (Figure 3L). The treatment did not modify the outcomes from the cue or context FC test (Figure 3M and Figure S2J, left plot). In female mice, Combination 2 seemed to negatively impact the memory performance of APP KI mice, as indicated by the lower percentage of alternations in the Y Maze and discrimination index in the NOR test (Figure 3N and 3O for the Y Maze and NOR test, respectively). FC cue memory was not influenced by the Combination 2 treatment in APP KI female mice (Figure 3P), nor was it in male mice. On the contrary, APP KI female mice treated with this regimen did not show a relevant increase in freezing behavior on Day 2 of the context test compared to Day 1, which was observed in the APP KI control group, suggesting a negative effect of this combination on associative learning and memory (Figure S2J, right plot).

comparison test: APP ctrl vs APP Comb 1 $p = 0.0259$) and percentage of time spent exploring the OF periphery in the first 10 min (one-way ANOVA, $F = 6.57$, $p = 0.0044$, followed by Tukey's multiple comparison test: APP ctrl vs APP Comb 1 $p = 0.0031$). (C) Latency to fall off the rotor measured in the three trials of Rotarod test. Repeated-measures two-way ANOVA, effect of group, $F = 8.86$, $p = 0.0011$, followed by Tukey's multiple comparison test: WT vs APP ctrl in Trial 1 $p = 0.0201$, WT vs APP ctrl in Trial 3 $p = 0.0056$, and APP ctrl vs APP Comb 1 in Trial 3 $p = 0.0018$. * $p < 0.05$ and ** $p < 0.01$ when comparing WT vs APP ctrl; ## $p < 0.01$ when comparing APP ctrl vs APP Comb 1. (D–F) The same test parameters as for male mice in A–C were analyzed in female mice from Cohort 1. (D) Repeated-measures two-way ANOVA, effect of group, $F = 5.09$, $p = 0.0133$, followed by Tukey's multiple comparison test: WT vs APP Comb 1 at 10 and 20 min intervals, $p = 0.0040$ and $p = 0.0034$, respectively. To the right, representative pattern of movement for WT, APP ctrl, or APP Comb 1 female mice. (E) Left plot: one-way ANOVA, $F = 4.60$, $p = 0.0191$, followed by Tukey's multiple comparison test: WT vs APP Comb 1 $p = 0.0165$. (G–H) Percentage of exploration time in open arms of EPM over the total 5 min trial duration in male mice (one-way ANOVA, $F = 3.76$, $p = 0.0357$, followed by Tukey's multiple comparison test: APP ctrl vs APP Comb 1 $p = 0.0446$), and in female mice (one-way ANOVA, $F = 6.30$, $p = 0.0061$, followed by Tukey's multiple comparison test: WT vs APP ctrl $p = 0.0183$ and WT vs APP Comb 1 $p = 0.0066$). To the right, representative heatmaps of EPM exploration, with warm colors showing the areas most explored by the animals. (I–J) Percentage of immobility time in FST test measured in the last 4 min of the session, in male and female mice. (K–M) Analysis of cognitive tests in WT, APP ctrl, and APP Comb 1 male mice from Cohort 1. (K) Percentage of spontaneous alternations calculated in the 5-min trial of the Y maze task. Red dashed line indicates the 50% chance level. One-sample *t*-test, $t = 2.79$, $p = 0.0317$, and $t = 2.82$, $p = 0.0201$ for the WT and APP Comb 1 groups, respectively). (L) Discrimination Index (DI) of novel vs familiar object exploration time during Day 3 of the NOR test, calculated as (N-F)/(N+F); N = novel and F = familiar object. Red dashed line represents DI = 0, with positive DI indicating preference for the novel object. (M) Percentage of freezing time during the cue FC test, comparing the time (2 min) before and during acoustic cue delivery. Repeated-measures two-way ANOVA, effect of time, $F = 34.57$, $p < 0.0001$, followed by Tukey's multiple comparison test: no sound vs sound $p = 0.0004$ and $p = 0.0137$ for WT and APP Comb 1 group, respectively. To the right, representative activity graphs of WT, APP ctrl, and APP Comb 1 male mice before and during sound. The graph highlights a clear reduction of activity during sound delivery compared to before, in WT and APP Comb 1 mice, but not in APP ctrl mouse. (N–P) The same test parameters as for male mice in K–M were analyzed for female mice from Cohort 1. (O) One-sample *t*-test, $t = 4.82$, $p = 0.0029$, $t = 3.88$, $p = 0.0031$, and $t = 3.62$, $p = 0.0040$ for WT, APP ctrl, and APP Comb 1 groups, respectively. (*p*) Repeated-measures two-way ANOVA, effect of time, $F = 424.6$, $p < 0.0001$, followed by Tukey's multiple comparison test: no sound vs sound $p < 0.0001$ for WT, APP ctrl, and APP Comb 1 groups. All data are presented as mean ± SEM. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $N = 10$ to 12 mice per group. Test illustrations were created with BioRender.com. APP, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in; DI, discrimination index; EPM, elevated plus maze; FST, forced swimming test; FC, fear conditioning; NOR, novel object recognition; N, number of mice; OF, open field; p, P value; SEM, standard error of the mean; WT, wild type.

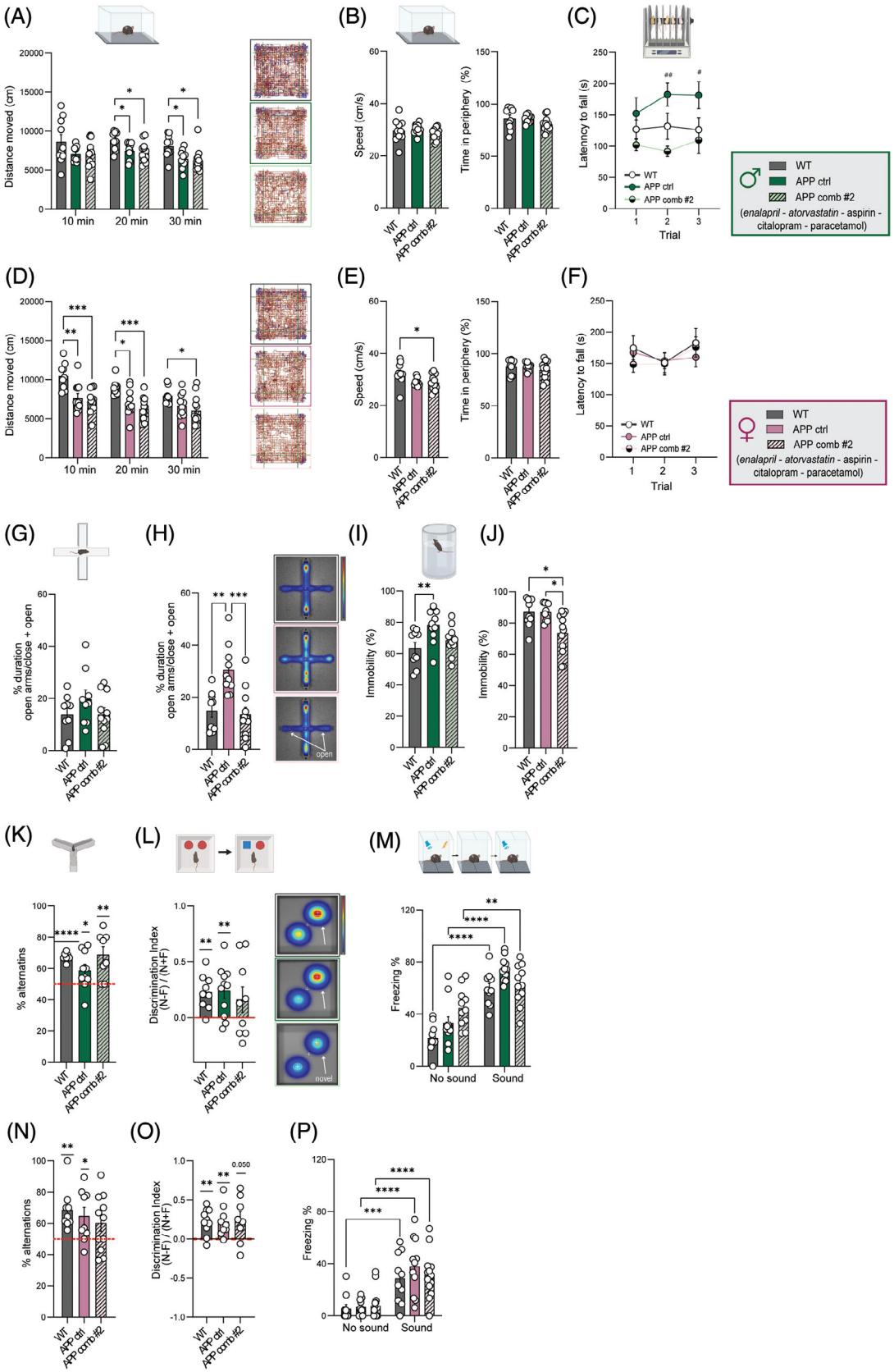


FIGURE 3 Effects of Combination 2 on behavioral outcomes in APP KI male and female mice. (AC) OF test analysis in WT, APP ctrl, and APP Comb 2 male mice from Cohort 2. (A) Distance moved measured over three 10 min time intervals. Repeated-measures two-way ANOVA, effect of group, $F = 5.88$, $p = 0.0070$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 2 at 20 min interval $p = 0.0330$ and $p = 0.0214$, respectively; WT vs APP ctrl and WT vs APP Comb 2 at 30 min interval $p = 0.0267$ and $p = 0.0216$, respectively. To the right, representative pattern of movements of the total 30 min trial for WT, APP ctrl, or APP Comb 2 male mice. (B) Average locomotor speed and

3.4 | Monotherapies exert sex-specific effects on different behavioral domains

Next, we aimed to explore whether selected monotherapies alone (citalopram, metoprolol, and simvastatin) could affect the behavioral outcomes investigated previously with multi-medication treatments. In APP KI male mice treated with citalopram, the distance traveled during the first 10 min in OF was lower when compared to WTs (Figure 4A), but the treatment did not affect the overall distance moved (Figure S2C, left plot). The drug treatments did not significantly influence the average speed, but citalopram-treated APP KI mice spent less time exploring the periphery of the OF arena compared to their control group (Figure 4B). In the Rotarod test, we observed that the main change was again in the citalopram group, which displayed a lower latency to fall than APP KI controls in Trial 3 (Figure 4C) and on average (Figure S2F, left plot). Different from male mice, we observed some opposite outcomes in female mice depending on the specific monotherapy diet. From the analysis of horizontal activity in OF, we found that citalopram further reduced the distance moved and speed in APP KI female mice compared to WTs, whereas metoprolol exerted an opposite effect by increasing the same parameters in APP KI mice (Figure 4D and E left plot, and Figure S2C, right plot). As for the previous cohorts, motor coordination assessed by Rotarod was not affected by genotype or any of the drug treatments in female mice (Figure 4F and Figure S2F, right plot).

When analyzing the outcomes from anxiety- and depressive-like behavior tasks, the main effect in male APP KI mice was detected in the citalopram group, which exhibited a lower time spent in the open arms of EPM compared to the APP KI control group (Figure 4G). In the same test, no effect was provoked by monotherapy diets in female mice (Figure 4H) or in the FST in both sexes (Figure 4I and J).

The assessment of spatial working memory in the Y Maze test showed that APP KI male mice administered the metoprolol diet had a higher significance in the percentage of alternations than the APP KI control group when compared to the 50% chance level (Figure 4K). Conversely, the APP KI simvastatin and citalopram groups did not alternate above the chance level on average, revealing a lower performance than the other groups in this test. When running the same test with female mice, we discovered that the metoprolol did not exert a positive effect. Instead, the metoprolol and citalopram groups poorly alternated the Y Maze arms, whereas simvastatin treatment positively influenced APP KI performance by increasing the percentage of alternations (Figure 4L). FC test outcomes did not reveal an effect of monotherapies in either sex (Figure 4M and N, and Figure S2K).

3.5 | Combination 1 decreases Aβ plaque load and amyloidogenic enzymes in the brains of APP KI male but not female mice

The APP KI mouse model already shows extensive amyloid pathology and gliosis in the cortex and hippocampus by the age of 6 months.[31,44] We decided to investigate whether the effects observed during behavioral assessment might relate to an effect of the treatments on amyloid pathology. We found widespread amyloidosis in the cortex and hippocampus of APP KI mice of both sexes, with accumulation of microglia around Aβ plaques (Figure 5A, and high magnification panel in Figure 5B). The analysis of Aβ deposits in the cortex revealed

percentage of time spent exploring the OF periphery in the first 10 mi. (C) Latency to fall off the rotor was measured in the three trials of Rotarod test. Repeated-measures two-way ANOVA, effect of group, $F = 12.00$, $p < 0.0001$, followed by Tukey's multiple comparison test: APP ctrl vs APP Comb 2 in Trials 2 and 3, $p = 0.015$, and $p = 0.0149$, respectively. # $p < 0.05$ and ## $p < 0.01$, when comparing APP ctrl vs APP Comb 2. (D–F) The same test parameters as for the male mice in A–C were analyzed in female mice from Cohort 2. (D) Repeated-measures two-way ANOVA, effect of group, $F = 11.02$, $p = 0.0003$, followed by Tukey's multiple comparison test: WT vs APP ctrl at 10 and 20 min intervals, $p = 0.0066$ and $p = 0.0140$, respectively; WT vs APP Comb 2 at 10 , 20 , and 30 min intervals, $p = 0.0010$, $p = 0.0002$, and $p = 0.0207$, respectively. To the right, representative pattern of movement for WT, APP ctrl, or APP Comb 2 female mice. (E) Left plot: one-way ANOVA, $F = 4.20$, $p = 0.0253$, followed by Tukey's multiple comparison test: WT vs APP Comb 2 $p = 0.0305$. (G and H) Percentage of exploration time in open arms of EPM over the total 5 min trial duration in male mice, and in female mice (one-way ANOVA, $F = 11.89$, $p = 0.0002$, followed by Tukey's multiple comparison test: WT versus APP ctrl $p = 0.0012$ and APP ctrl vs APP Comb 2, $p = 0.0002$). (I and J) Percentage of immobility time in FST test measured in the last 4 min of the session, in male mice (one-way ANOVA, $F = 5.70$, $p = 0.0080$, followed by Tukey's multiple comparison test: WT vs APP ctrl, $p = 0.0070$) and in female mice (one-way ANOVA, $F = 6.72$, $p = 0.0040$, followed by Tukey's multiple comparison test: WT vs APP Comb 2 and APP ctrl vs APP Comb 2, $p = 0.0106$ and $p = 0.0111$). (K–M) Analysis of cognitive tests in WT, APP ctrl, and APP Comb 1 male mice from Cohort 2. (K) Percentage of spontaneous alternations calculated in the 5 min trial of the Y maze task. Red dashed line indicates the 50% chance level. One-sample *t*-test, $t = 16.65$, $p < 0.0001$, $t = 2.32$, $p = 0.0456$, and $t = 3.67$, $p = 0.0080$ for WT, APP ctrl, and APP Comb 2 groups, respectively. (L) DI of novel vs familiar object exploration time during Day 3 of NOR test. Red dashed line represents DI = 0. One-sample *t*-test, $t = 4.38$, $p = 0.0024$, $t = 3.52$, $p = 0.0056$ for WT and APP ctrl groups, respectively. (M) Percentage of freezing time during the cue FC test, comparing the time (2 min) before and during acoustic cue delivery. Repeated-measures two-way ANOVA, effect of time, $F = 99.69$, $p < 0.0001$, followed by Tukey's multiple comparison test: no sound vs sound $p < 0.0001$ for WT and APP ctrl group, respectively, and $p = 0.0092$ for the APP Comb 2 group. (N–*p*) The same test parameters as for male mice in (K–M) were analyzed for female mice from Cohort 2. (N) One-sample *t*-test, $t = 4.21$, $p = 0.0029$, $t = 2.68$, $p = 0.0280$ for WT and APP ctrl groups, respectively. (O) One-sample *t*-test, $t = 4.25$, $p = 0.0021$, $t = 3.77$, $p = 0.0044$ for WT and APP ctrl groups, respectively. (P) Repeated-measures two-way ANOVA, effect of time, $F = 88.83$, $p < 0.0001$, followed by Tukey's multiple comparison test: no sound vs sound $p = 0.0002$ for WT group, and $p < 0.0001$ for the APP ctrl and APP Comb 2 groups, respectively. All data are presented as mean ± SEM. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $N = 10$ to 13 mice per group. Test illustrations were created with BioRender.com.APP, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in; DI, discrimination index; EPM, elevated plus maze; FST, forced swimming test; FC, fear conditioning; NOR, novel object recognition; OF, open field; SEM, standard error of the mean; WT, wild type; *N*, number of mice; *p*, *p* value.

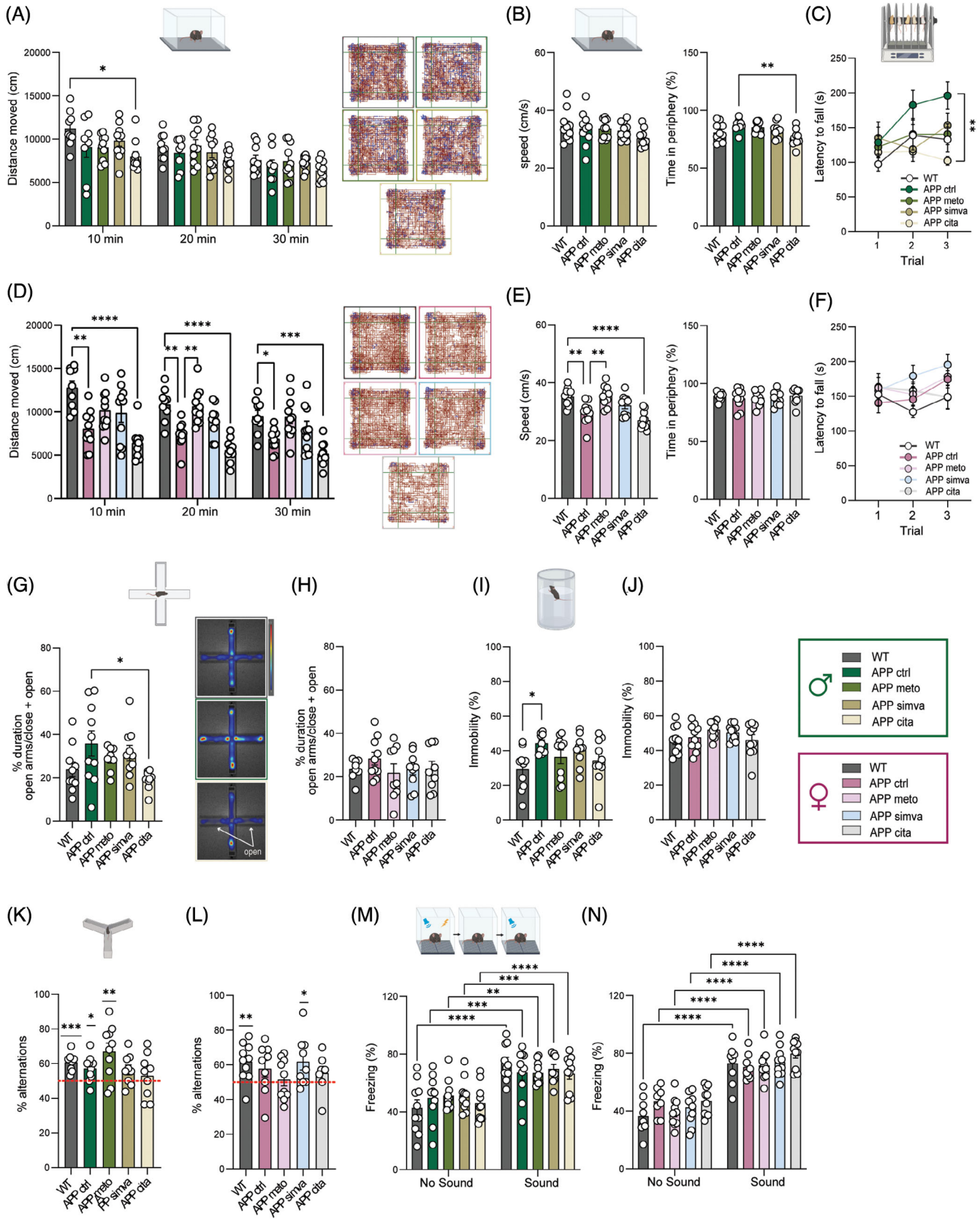


FIGURE 4 Effects of monotherapies on behavioral outcomes in APP KI male and female mice. (A–C) OF test analysis in WT, APP ctrl, APP meto, APP simva, and APP cita male mice from Cohort 3. (A) Distance moved measured over three, 10 min time intervals. Repeated-measures two-way ANOVA, effect of group, $F = 1.86$, $p = 0.1352$, followed by Tukey's multiple comparison test: WT vs APP cita at 10-min interval $p = 0.0159$. To the right, representative pattern of movements of the total 30 min trial for WT, APP ctrl, and APP monotherapy male mice. (B) Average locomotor speed and percentage of time spent exploring the OF periphery in the first 10 min (one-way ANOVA, $F = 4.13$, $p = 0.0063$, followed by

that the number and area of amyloid plaques were reduced in APP KI male mice treated with Combination 1 compared to their APP KI controls (high magnification panel and top plots in Figure 5B for ROI i, and Figure S3A, for ROI ii), but not in female mice (Figure 5B, bottom panels, and Figure S3B). Combination 2 did not change Aβ load in either sex (Figure 5C and D for ROI i, and Figure S3C and D, for ROI ii). We also analyzed the levels of cortical soluble and insoluble fractions of Aβ42 and found a nonsignificant decreasing trend in the amount of insoluble Aβ42 in Combination 1–fed APP KI male mice compared to APP KI control mice (Figure 5E, right plot), which correlated positively with our findings of lower Aβ deposits in the cortex from the immunostaining experiments. The soluble fraction of Aβ42 remained unchanged in this cohort (Figure 5E, left plot). The same multi-medication treatment did not affect Aβ42 levels in APP KI female mice (Figure 5F), and no effect was observed when analyzing the same proteins in the cortex of APP KI male and female mice administered a Combination 2 diet (Figure 5G and H). When examining the data from our monotherapy cohorts, we saw that the levels of soluble Aβ42 were significantly lower in the metoprolol and citalopram APP KI male groups (Figure 5I), whereas a similar effect was exerted by citalopram in APP KI female mice but on insoluble Aβ42 fraction levels (Figure 5J). We also analyzed the levels of soluble and insoluble Aβ40, without detecting any relevant difference between treated and control groups across all the animal cohorts (Figure S3E–J), apart from a decrease of Aβ40 insoluble fraction in Combination 2–treated APP KI female mice than controls (Figure S3I).

We hypothesized that alterations of APP processing or the enzymes involved in the amyloidogenic pathway could contribute to fewer Aβ plaques observed in male mice treated with Combination 1. Therefore, we measured the levels of total APP in the brains of APP KI male mice. In Combination 1–treated APP KI mice, the levels of full-length APP were not significantly changed compared to the control group, whereas citalopram alone reduced the amount of this protein in APP KI male mice (Figure 5K). *App*, *Bace1*, *Psen1*, and *Adam10* gene expression were quantified in the hippocampus of WT and APP KI mice from Combination 1 cohorts. These are crucial enzymes in the amyloidogenic pathway, and modifying their activity might lead to an alteration of pathological Aβ production. We saw a reduction of *Bace1* and *Psen1* in APP KI male mice treated with the multi-medication diet 1 compared to their control group (Figure 5L), whereas no differences were found in female mice (Figure 5M). The same genes were also analyzed in monotherapy-treated cohorts, but when the drugs were administered alone, we did not observe any significant change in APP KI groups of either sex (Figure S3K–R).

3.6 | Combination 1 modifies the expression levels of homeostatic and reactive microglia in the hippocampus of APP KI male but not female mice

Given the key role of microglia in amyloid pathology, we wanted to evaluate whether changes in the levels of microglial markers could support the observed reduction of Aβ plaques in Combination 1–treated male mice. As expected, due to the extensive amyloid deposition, the levels of homeostatic and phagocytic microglial markers *Tmem119*, *Aif1*, *Trem2*, and *Clec7a* were increased in the hippocampus of APP KI male mice compared to WTs (Figure 6A–E). Of interest, Combination 1 treatment lowered *Tmem119* and *Aif1* in APP KI male mice to the level of WTs (Figure 6A and B), and decreased *Clec7a* (Figure 6E). We also measured some other markers for immune activation and inflammatory responses, such as *CD68*, *Ifit3*, and *Irf7*, which were also upregulated in APP KI male mice, without a significant effect induced by the multiple-drug regimen (Figure 6F–H). The reduction of *Tmem119*, *Aif1*, and *Clec7* by Combination 1 in APP KI male mice was sex-specific, as we did not find any relevant changes in the expression of those genes in APP KI female mice (Figure 6J–Q), in line with the fact that treated females did

Tukey's multiple comparison test: APP ctrl vs APP cita $p = 0.0056$). (C) Latency to fall off the rotor measured in the three trials of Rotarod test. Repeated-measures two-way ANOVA, effect of group, $F = 2.29$, $p = 0.0742$, followed by Tukey's multiple comparison test: APP ctrl vs APP cita in Trial 3 $p = 0.0061$. (D–F) The same test parameters as for males in A–C were analyzed in female mice from Cohort 3. (D) Repeated-measures two-way ANOVA, effect of group, $F = 13.17$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl at 10 , 20 , and 30 min intervals, $p = 0.0027$, $p = 0.0042$, and $p = 0.0326$, respectively; WT vs APP cita at 10- and 20-min intervals, $p < 0.0001$, and at 30 min interval $p = 0.0007$, respectively; APP ctrl vs APP meto at 20 min interval $p = 0.0075$. To the right, representative pattern of movements for WT, APP ctrl, and APP monotherapy female mice. (E) Left plot: one-way ANOVA, $F = 10.87$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl, WT vs APP cita, and APP ctrl vs APP meto $p = 0.0073$, $p < 0.0001$, and $p = 0.0043$, respectively. (G and H) Percentage of exploration time in open arms of EPM over the total 5 min trial duration in male mice (one-way ANOVA, $F = 3.05$, $p = 0.0272$, followed by Tukey's multiple comparison test: APP ctrl vs APP cita $p = 0.0233$), and in female mice. (I and J) Percentage of immobility time in FST test measured in the last 4 min of the session in male mice (one-way ANOVA, $F = 2.75$, $p = 0.0397$, followed by Tukey's multiple comparison test: WT vs APP ctrl $p = 0.0294$) and in female mice. (K and L) Percentage of spontaneous alternations calculated in the 5 min trial of the Y maze task in male mice (one-sample *t*-test, $t = 5.63$, $p = 0.0008$, $t = 3.07$, $p = 0.0133$, and $t = 3.50$, $p = 0.0067$ for WT, APP ctrl, and APP meto groups, respectively) and in female mice (one-sample *t*-test, $t = 3.44$, $p = 0.0074$, $t = 2.49$, $p = 0.0378$ for WT and APP simva groups, respectively). Red dashed line indicates the 50% chance level. (M and N) Percentage of freezing time during the cue FC test, comparing the time (2 min) before and during acoustic cue delivery, in male mice (repeated-measures two-way ANOVA, effect of time, $F = 137.00$, $p < 0.0001$, followed by Tukey's multiple comparison test: no sound vs sound $p < 0.0001$ for WT and APP cita groups, respectively, and $p = 0.0003$, $p = 0.0013$, and $p = 0.0001$ for APP ctrl, APP meto, and APP simva groups, respectively) and in female mice (repeated-measures two-way ANOVA, effect of time, $F = 436.1$, $p < 0.0001$, followed by Tukey's multiple comparison test: no sound vs sound $p < 0.0001$ for all groups). All data are presented as mean ± SEM. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $N = 10$ mice per group. Test illustrations were created with BioRender.com. APP, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in; DI, discrimination index; EPM, elevated plus maze; FST, forced swimming test; FC, fear conditioning; OF, open field; SEM, standard error of the mean; WT, wild type.

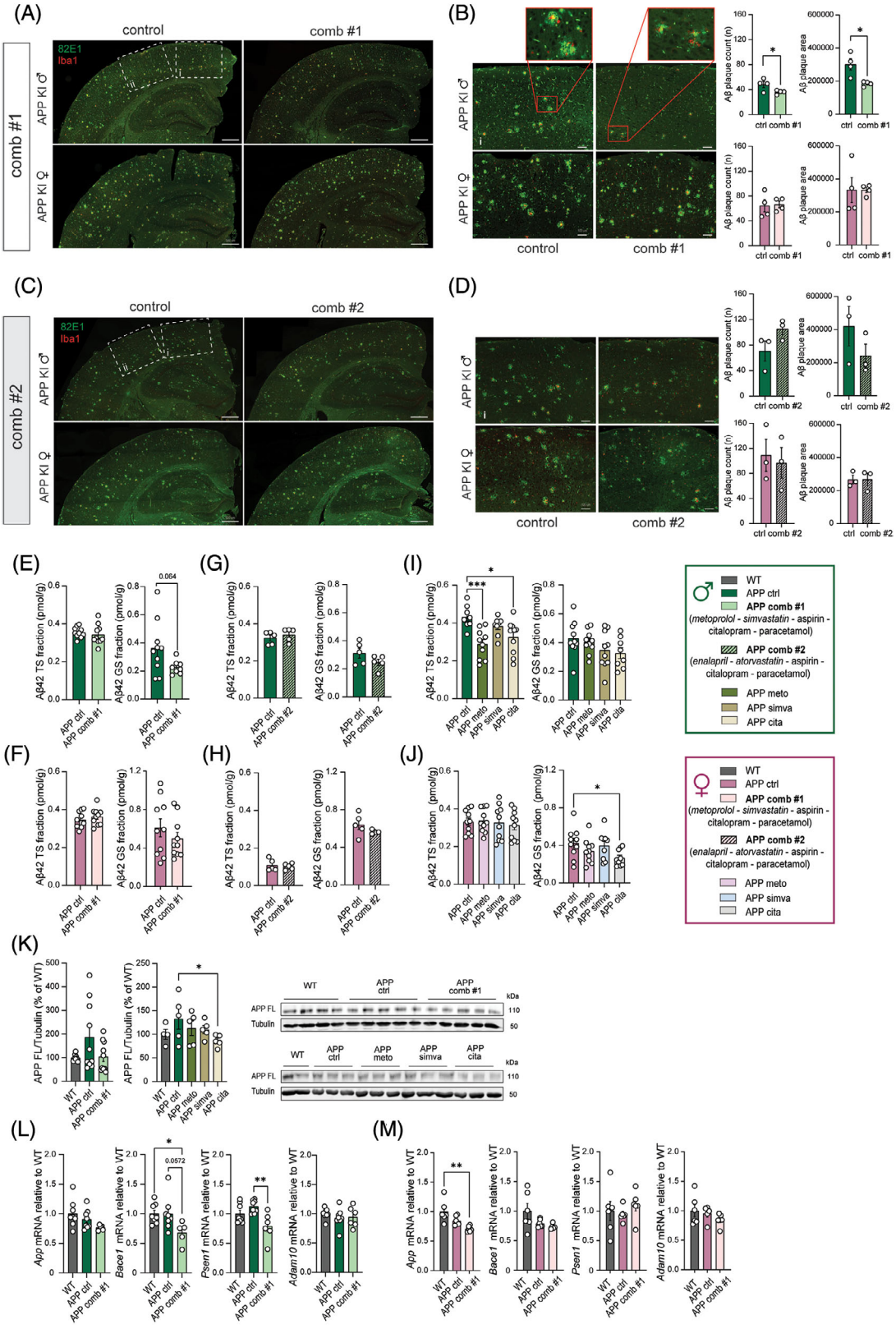


FIGURE 5 Effects of multi-medication treatments and monotherapies on amyloid pathology in APP KI male and female mice. Representative images of immunofluorescent staining in cortico-hippocampal areas from coronal brain slices with anti-Aβ 82E1 in green and anti-Iba1 in red from APP KI mice treated with Combination 1 (A and B), or Combination 2 (C and D) of both sexes. White dashed rectangles indicate the regions of interest (ROIs) used for the quantification analysis. Magnifications of cortical ROIs are displayed in the panels in (B) with further enlargement of Aβ plaque example from APP ctrl and APP Comb 1 male mice, and in (D) for APP ctrl and APP Comb 2 mice. To the right, the bar plots show the analysis of Aβ plaque number and area from the ROIs (i). Unpaired *t*-test, $t = 2.40$, $p = 0.0531$ and $t = 3.41$, $p = 0.0143$ for Aβ plaque count and area, respectively, in APP ctrl vs APP Comb 1 male mice. $N = 3$–4 mice per group. Scale bar = 500 um in A and C, and 100 um in B and D. Quantification of

not show reduction of Aβ plaques. Iba1 and GFAP were also measured as general markers for microglia and astrocytes, revealing a decreasing tendency of Iba1 levels in APP KI male mice treated with Combination 1, which was not seen for GFAP (Figure 6I). In APP KI female mice, polypharmacy Combination 1 did not alter Iba1 amount, whereas GFAP further increased in the treatment group compared to the controls (Figure 6R). To determine whether some specific medications could drive some of the effects caused by Combination 1 on microglia markers, we ran the same experiments in the brains of monotherapy cohort animals. We found that neither metoprolol nor simvastatin or citalopram alone influenced the levels of inflammation-related genes (Figure S4A–H). GFAP levels were higher in APP KI male mice treated with simvastatin than the APP KI controls (Figure S4I). In APP KI female mice fed with monotherapies, the marker for homeostatic microglia *P2ry12* was higher in the citalopram than in the control group (Figure S4L), whereas no other effect was detected in the other single drug groups (Figure S4J–Q). Although we did not see an effect of Combination 1 on the levels of Iba1 in APP KI female mice, simvastatin and citalopram diminished the levels of this protein when administered alone (Figure S4R). The same western blotting analysis also showed a reduction of GFAP by simvastatin in APP KI female mice compared to controls. These results might indicate a sex-specific protective effect of Combination 1 on neuro-inflammation in male mice, alongside the observed reduction of amyloid load, which is not visible to the same extent with the administration of single medications. In female mice, simvastatin and citalopram showed more effects than in male mice when administered alone.

A summary of the effects of multi-medication and monotherapy regimens on behavioral and molecular biology outcomes in APP KI male and female mice is represented in the heatmaps in Figure 7A and B.

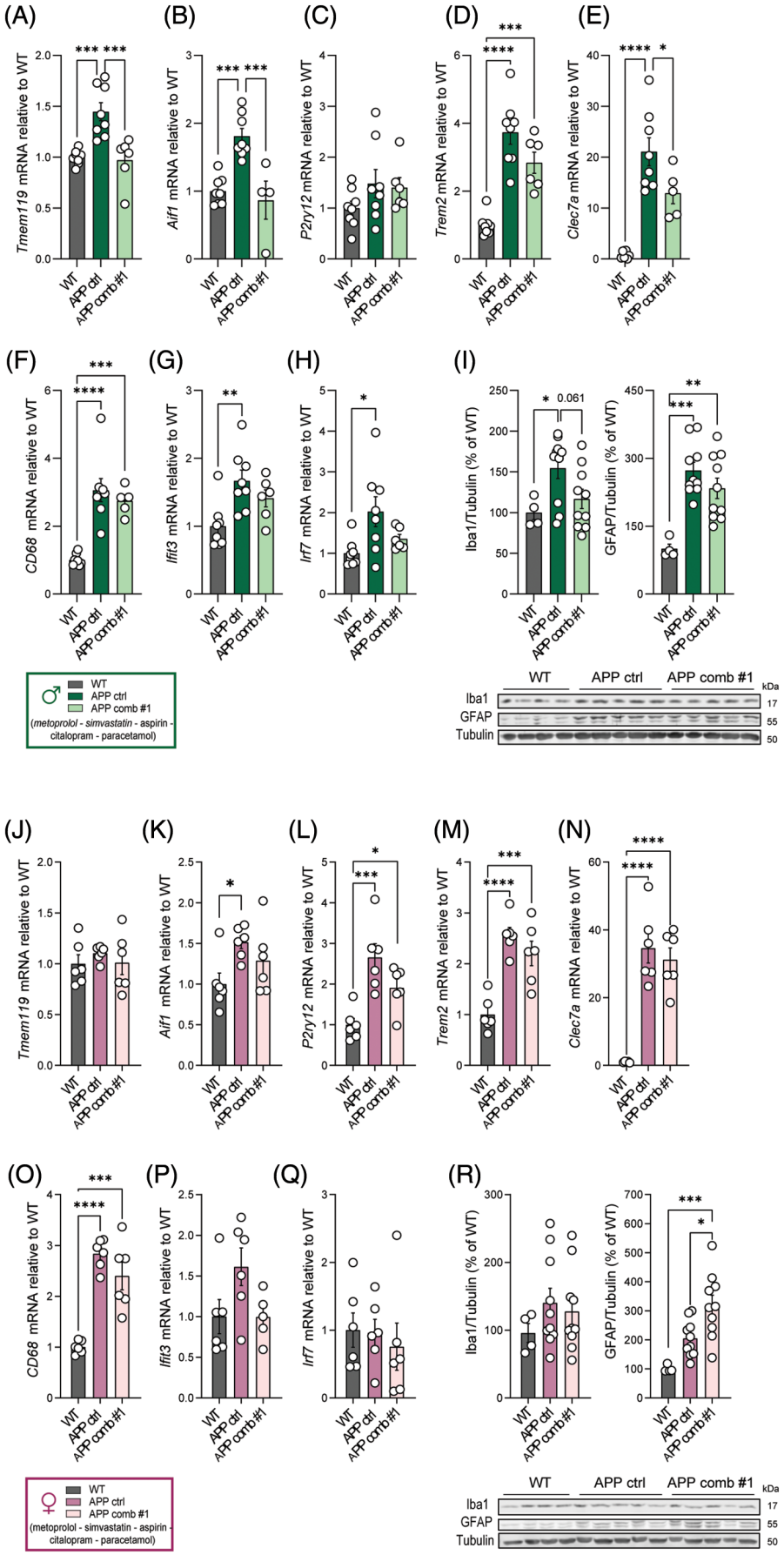
3.7 | Multi-medication therapies alter serum metabolome differently in APP KI male and female mice, with Combination 1 positively modulating AD-related metabolomic profiles in male mice

To gain further insights into the impact of drug treatments on AD pathology, we performed targeted metabolomics analysis on the serum collected from Combinations 1 and 2 mouse cohorts after 8 weeks of treatment. A total of 309 metabolic features were identified in both sexes. In male mice, PCA revealed a clear separation of the Combination 1–treated APP KI group from the control groups, especially along the first principal component (PC1, 24.7% of total variance, Figure 8A). In contrast, WT and APP KI control groups clustered closely together. In female mice, we observed a more evident separation by genotype, as WT and APP KI control groups were more segregated from each other, particularly along the PC1 axis (PC1, 22.7% of total variance, Figure 8B). In this cohort, the Combination 1–treated APP KI group lay between the other two, suggesting lower variation between the treated group and the controls. The evident cluster of samples from APP KI male mice administered Combination 1 suggests a stronger treatment effect by this combination in male than in female mice (Figure 8A). The analysis of differential metabolites in treated APP KI mice compared to APP controls showed that Combination 1 led to a significant alteration of 40 and 37 metabolites in male and female mice, respectively (volcano plots, Figure 8C and D). In male mice, 24 metabolites were downregulated by the treatment, and 16 were upregulated. Among the changed metabolites in male mice, we found serotonin, L-kynurenine, lysophospholipids, and steroids, such as cholesterol and beta-sitosterol (Table S2). In contrast, in female mice, a large portion of metabolites altered by the multi-medication regimen 1 belonged to carnitines and fatty acids (Table S3). Serotonin, adenosine, and some metabolites from carnitine and fatty acid groups were commonly altered in both sexes. Noteworthy, although Combination 1 increased the levels of carnitines in APP KI male mice, in female mice this class was strongly downregulated. We performed MSEA to acquire information on potential pathways that could be differently regulated by the multiple-drug combinations, beyond the individual metabolite changes. Some of the significantly enriched pathways identified by MSEA in male mice were gluconeogenesis/glycolysis, purine metabolism, and tryptophan metabolism (Figure S5A). These processes were not significantly altered in female mice by the same multidrug diet. The most enriched pathway in female mice was alpha-linolenic acid and linoleic acid metabolism (Figure S5B).

In Combination 2 cohorts, PCA did not reveal a clear distinction between groups by treatment or genotype, especially in female mice (Figure 8E and F, for male and female groups, respectively). In male mice, although the WT and APP KI–treated group appeared to have a broader distribution along the PC2 axis and were slightly

Aβ42 TS (soluble) and GS (insoluble) cortical fractions in male and female APP KI mice treated with Combination 1 (E and F), Combination 2 (G and H), and monotherapies (I and J), in male mice, one-way ANOVA, $F = 6.84$, $p = 0.0011$, followed by Tukey's multiple comparison test: APP ctrl vs APP meto and APP ctrl vs APP cita, $p = 0.0006$ and $p = 0.0105$, respectively; and in female mice, one-way ANOVA, $F = 2.39$, $p = 0.0862$, followed by Tukey's multiple comparison test: APP ctrl vs APP cita, $p = 0.0473$. $N = 10$ mice per group in Combination 1 and monotherapy cohorts, respectively, and $N = 5$ mice per group in Combination 2 cohort. (K) Quantification analysis and representative images of western blot experiments on TS cortical lysates of APP full length (APP FL) protein in Combination 1 and monotherapy male cohorts. One-way ANOVA, $F = 1.57$, $p = 0.2220$, followed by Tukey's multiple comparison test: APP ctrl vs APP cita, $p = 0.0258$. $N = 5$–10 mice per group. (L and M) Expression of *App*, *Bace1*, *Psen1*, and *Adam10* in the hippocampus of APP KI male and female mice administered Combination 1 (in male mice: one-way ANOVA, for *Bace1* $F = 3.87$, $p = 0.0399$, followed by Tukey's multiple comparison test: WT vs APP Comb 1, $p = 0.0535$ and APP ctrl vs APP Comb 1, $p = 0.0572$; and for *Psen1*, $F = 6.73$, $p = 0.0062$, followed by Tukey's multiple comparison test: APP ctrl vs APP Comb 1, $p = 0.0045$. In female mice: one-way ANOVA, for *App* $F = 8.79$, $p = 0.0030$, followed by Tukey's multiple comparison test: WT vs APP Comb 1, $p = 0.0021$). $N = 6$–8 per group. All data are presented as mean ± SEM. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. APP, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in; ROI, region of interest; SEM, standard error of the mean; TS, tris-soluble; GS, guanidine-soluble; WT, wild type.

Alzheimer's & Dementia®
THE JOURNAL OF THE ALZHEIMER'S ASSOCIATION



separated from each other (PC2, 15.4% of total variance, Figure 8E), all three groups showed some degree of overlap. We identified a lower number of differentially regulated metabolites by Combination 2 in both sexes compared to the previous polypharmacy regimen. In male mice, 25 metabolites were significantly changed in treated APP KI serum samples compared to APP controls (volcano plot, Figure 8G), including serotonin and lysophospholipids, which were also altered by Combination 1 in the same sex, together with some metabolites from nucleosides and fatty acid classes (Table S4). In female mice, only 13 metabolic features were significantly altered by the treatment (volcano plots, Figure 8H), some of which appeared to be shared with male mice (Table S5). MSEA detected that the purine metabolism pathway was commonly enriched in both sexes (Figure S5C and D), whereas the regulation of other pathways, such as aspartate metabolism, was sex-specific. Despite MSEA analysis identifying several metabolic pathways modulated by the multiple-drug diets, only a small number reached statistical significance. We suggest that the relatively limited number of metabolites analyzed in this study through the targeted approach may have contributed to lowering the statistical power in the analysis of enriched pathways. The results from metabolomics analysis indicate that Combination 1 more significantly affects metabolomic profiles in male APP KI mice, consistent with the outcomes from cognitive and molecular biology experiments, whereas Combination 1 showed more prominent effects in male mice and overall than Combination 2.

4 | DISCUSSION

Polypharmacy is prevalent among older adults and people with ADRD.[21] Although associated with adverse outcomes, medications frequently used in polypharmacy therapies target dementia risk factors, offering potential benefits in preventing or mitigating ADRD disorders. However, no previous experimental study has examined the effects of polypharmacy in the presence of these conditions. This led us to investigate how commonly used multiple-drug treatments affect AD onset and progression in the $App^{NL\text{-}G\text{-}F}$ mouse model, considering the impact of sex. Our results show that one specific drug combination (metoprolol, simvastatin, aspirin, paracetamol, and citalopram) improved some measures of cognitive performance, decreased locomotion and anxiety-like behavior, and reduced amyloid plaques in male mice but did not affect these parameters in female mice. Replacing metoprolol and simvastatin with enalapril and atorvastatin eliminated these effects in male mice. In female mice, the substituted combination reduced anxiety-like behavior but impaired memory performance. Sex differences were evident not only with drug combinations but also with monotherapies, and may be attributed to factors such as pharmacokinetics and pharmacodynamics, including sex-specific drug interactions, hormonal effects on the brain, variations in receptor distribution, gene expression, metabolic pathways, and ultimately, disease pathophysiology. As reported in WT mice,[22,23,45] Combination 1 and citalopram alone impacted functional and emotional domains, predominantly in male mice. In female mice, citalopram alone did not affect the OF, EPM, and FST tests. This could be attributed to hormonal fluctuations, which can modulate serotonin receptors. Estrogen affects the density and function of serotonin receptors, such as 5- HT1A and 5-HT2A subtypes, which are key targets of selective serotonin reuptake inhibitors (SSRIs) (like citalopram). As a result, variations in estrogen levels can lead to differential responses to antidepressant treatments between male and female mice.[46] In addition, the expression of serotonin receptor subtypes 5-HT1A and 5-HT2A varies between male and female mice.[47] These considerations highlight the importance of considering hormonal influences when evaluating therapeutic responses

FIGURE 6 Effects of Combination 1 on the expression levels of glial markers in APP KI male and female mice. (A–H) Expression levels of *Tmem119*, *Aif1*, *P2ry12*, *Trem2*, *Clec7a*, *CD68*, *Ifit3*, and *Irf7* in the hippocampus of APP KI male mice treated with Combination 1. (A) One-way ANOVA, $F = 13.75$, $p = 0.0002$, followed by Tukey's multiple comparison test: WT vs APP ctrl and APP ctrl vs APP Comb 1, $p = 0.0006$ and $p = 0.0008$, respectively. (B) One-way ANOVA, $F = 15.46$, $p = 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl and APP ctrl vs APP Comb 1, $p = 0.0005$ and $p = 0.0008$, respectively. (D) One-way ANOVA, $F = 27.88$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 1, $p < 0.0001$ and $p = 0.0006$, respectively. (E) One-way ANOVA, $F = 29.05$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl and APP ctrl vs APP Comb 1, $p < 0.0001$ and $p = 0.0388$, respectively. (F) One-way ANOVA, $F = 23.03$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 1, $p < 0.0001$ and $p = 0.0004$, respectively. (G) One-way ANOVA, $F = 6.51$, $p = 0.0070$, followed by Tukey's multiple comparison test: WT vs APP ctrl $p = 0.0054$. (H) One-way ANOVA, $F = 4.69$, $p = 0.0221$, followed by Tukey's multiple comparison test: WT vs APP ctrl $p = 0.0183$. (I) Quantification analysis and representative images of western blot experiments on TS cortical lysates of Iba1 and GFAP proteins in Combination 1 male mice. One-way ANOVA, for Iba1, $F = 4.18$, $p = 0.0297$, followed by Tukey's multiple comparison test: WT vs APP ctrl and APP ctrl vs APP Comb 1, $p = 0.0393$ and $p = 0.0611$, respectively; for GFAP, $F = 11.74$, $p = 0.0004$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 1, $p = 0.0003$, and $p = 0.0034$, respectively. (J and Q) Expression levels of *Tmem119*, *Aif1*, *P2ry12*, *Trem2*, *Clec7a*, *CD68*, *Ifit3*, and *Irf7* in the hippocampus of APP KI female mice treated with Combination 1. (K) One-way ANOVA, $F = 5.68$, $p = 0.0500$, followed by Tukey's multiple comparison test: WT vs APP ctrl $p = 0.0406$. (L) One-way ANOVA, $F = 11.06$, $p = 0.0011$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 1, $p = 0.0008$ and $p = 0.0514$, respectively. (M) One-way ANOVA, $F = 20.19$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 1, $p < 0.0001$ and $p = 0.0009$, respectively. (N) One-way ANOVA, $F = 26.86$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 1, $p < 0.0001$. (O) One-way ANOVA, $F = 30.55$, $p < 0.0001$, followed by Tukey's multiple comparison test: WT vs APP ctrl and WT vs APP Comb 1, $p \leq 0.0001$. (R) Quantification analysis and representative images of western blot experiments on TS cortical lysates of Iba1 protein and GFAP in Combination 1 female mice. One-way ANOVA, for GFAP, $F = 11.07$, $p = 0.0005$, followed by Tukey's multiple comparison test: WT vs APP Comb 1 and APP ctrl vs APP Comb 1, $p = 0.0006$ and $p = 0.0152$, respectively. All data are presented as mean ± SEM. $N = 6$–8 mice per group for qPCR experiments, and $N = 4$–10 mice per group for WB experiments. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. APP, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in; ROI, region of interest; SEM, standard error of the mean; -: WT, wild type.

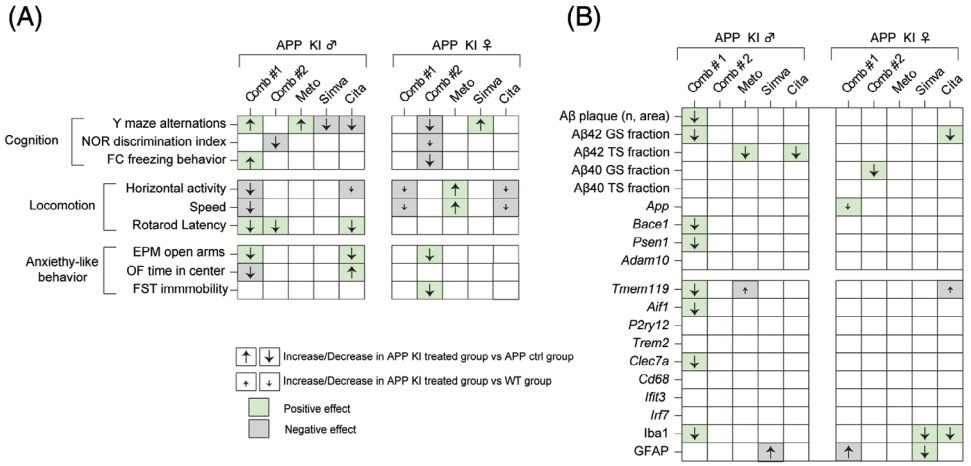


FIGURE 7 Summary of behavioral and molecular biology outcomes. (A) Representative heatmap summarizing the main changes from the analysis of behavioral outcomes in male and female cohorts (showed in Figures 2–4 and Figure S2). Arrows indicate significantly increased or decreased parameters measured in the tests for cognition, locomotion, and anxiety-like behavior, in APP KI–treated mice vs the APP KI ctrl or vs the WT group. (B) Representative heatmap summarizing the main changes from the analysis of molecular outcomes in male and female cohorts (shown in Figures 5 and 6, and Figures S3 and S4). Arrows indicate significantly increased or decreased levels of Aβ, APP processing enzymes, and neuroinflammatory markers in APP KI–treated mice vs the APP KI ctrl or vs the WT group. Green and gray rectangles highlight positive and negative effects of the treatment on the specific outcome. APP, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in; WT, wild type.

in women. Combination 2 (including citalopram, but not metoprolol) showed minor effects on locomotion. β-Blockers such as metoprolol can decrease physical activity when given in combinations, likely related to pharmacokinetic and pharmacodynamic interactions. This may account for the lack of effects in Combination 2, where metoprolol was absent. Metoprolol monotherapy improved cognition in male mice with no effect on locomotion, consistent with a previous study in WT aged male mice,[45] while having no effect on cognition and improving locomotion in female mice.[48,49] The pharmacokinetic interaction, whereby citalopram can increase the concentration of metoprolol, may partly explain these differences. Anxiety- and depressive-like behavior was positively affected by polypharmacy Combination 2, predominantly in female mice. Blocking the renin-angiotensin system components can reduce anxiety- and depressive-like behaviors in rodents.[50,51] Thus the presence of enalapril in Combination 2 might contribute to the differential effects shown on anxiety and depression tasks by this specific multiple-drug diet, which were less evident in Combination 1. Differences in the metabolism of ACE inhibitors, driven by sex-specific enzyme regulation,[52,53] may explain our findings in female mice.

Combination 1 had beneficial effects on spatial and fear-conditioned learning and memory in APP KI male mice, whereas monotherapies had diverse effects. Metoprolol positively affected male mice, but simvastatin and citalopram did not. The cognitive improvement by polypharmacy Combination 1 was accompanied by a reduction of Aβ plaques in APP KI male mice. Low-dose aspirin has been shown previously to ameliorate cognition and decrease amyloid plaques in AD mice,[54] and in patients with AD, aspirin use is associated with slower cognitive decline[55]. Aspirin may have contributed to the positive effects, although those were lost in Combination 2, where this compound was still present. Likewise, simvastatin rescues memory functions in AD rodents[56] and differentially affects functional outcomes in WT mice, depending on age and sex.[49] Simvastatin is also associated with better cognitive scores in people with dementia than atorvastatin,[34] which in mice is shown to induce cognitive and hippocampal dysregulation.[57] In our study, simvastatin showed good outcomes in female mice, while negatively influencing spatial memory in APP KI male mice. A reduction in Aβ42 levels was also found in the brains of APP KI male mice treated with metoprolol and citalopram alone. The role of β-blockers on amyloid pathology in AD is contradictory, with recent research showing increased brain clearance of Aβ and tau,[58] consistent with our results. On the other hand, chronic β-receptor blockade may potentiate neuroinflammation in models of amyloidosis.[59] A similar decrease in Aβ production has been reported in both healthy individuals and transgenic AD mice following antidepressant treatment with citalopram, suggesting that SSRIs may reduce Aβ42 levels via modulation of APP processing rather than directly targeting amyloid plaques.[60,61]

In line with this hypothesis, Combination 1 reduced β-secretase (*BACE1*) and γ-secretase (*PSEN1*) enzyme expression in APP KI male but not in female mice, potentially contributing to decreased Aβ production in male mice. We observed a reduction of the homeostatic microglial markers *Tmem119* and *Aif1*/Iba1, and the disease-associated microglial marker *Clec7a* in male but again not in female mice. The reduction of these markers might indicate a lower need for activated microglia, due to the reduction of amyloid burden induced by Combination 1. The lack of substantial effects by monotherapies in male mice on microglial signatures could indicate a synergistic effect of the drugs used in Combination 1.

Unlike in male mice, female APP KI showed more evident changes in molecular outcomes when administered monotherapies than multimedication diets. Citalopram reduced insoluble Aβ42 levels, and

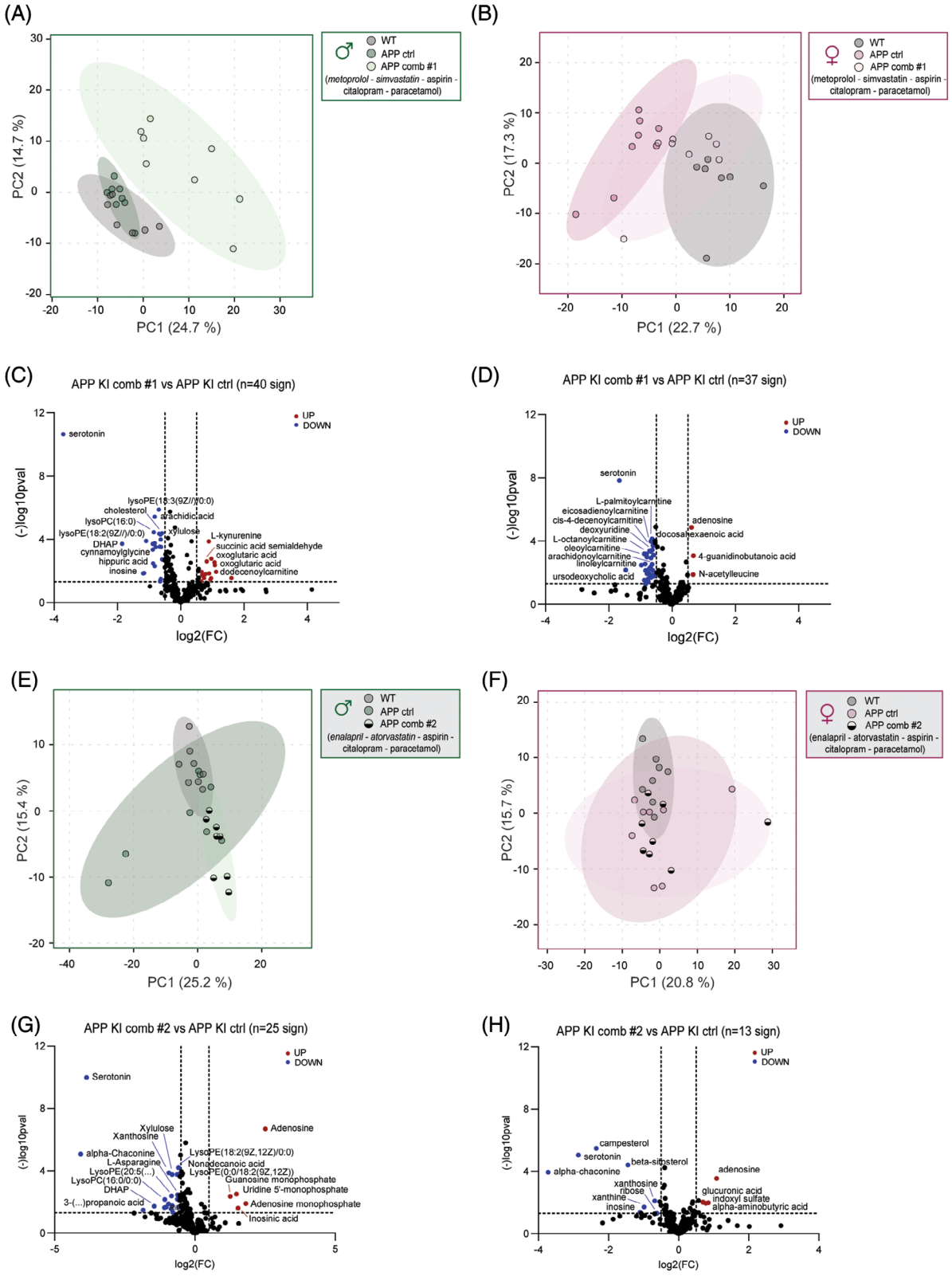


FIGURE 8 Serum metabolomics profiles in APP KI male and female mice treated with multi-medication regimens. (A–B and E–F) PCA of serum metabolites from male and female WT and APP KI mice administered Combination 1 or 2. (C–D and G–H) Volcano plot analysis displaying differentially regulated metabolites in male and female APP KI mice treated with Combination 1 or 2 vs the respective APP ctrl group. Red and blue dots indicate significantly upregulated and downregulated metabolites, $p < 0.05$ and ± 1.5 FC (± 0.58 log2 FC). $N = 7–8$ mice per group. APP, *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in; PCA, principal component analysis; WT, wild type.

simvastatin and citalopram decreased Iba1 and GFAPs in the cortex of APP KI female mice. Nevertheless, neither multi-medication regimen reduced amyloid pathology in APP KI female mice, with Combination 2 negatively impacting cognition in this sex. This may suggest the presence of antagonistic effects between drugs in the multi-medication treatments.

We conducted metabolomic analysis in WT and APP KI mice serum to identify metabolic targets associated with polypharmacy outcomes in AD pathology. Altered metabolic features showed limited overlap in treated male and female AD mice, indicating that sex differentially affects metabolic pathways in response to polypharmacy. Some of them were previously reported to be implicated in AD and with sex-specific changes.[62–64] However, common metabolite classes, such as amino compounds like serotonin, fatty acids, and carbohydrates, were altered in both sexes, to a different extent. PCA did not show a clear separation by genotype, especially in male mice. This was observed previously in an early-onset AD mouse model.[63] Lysophospholipids play an essential role in neural membrane formation and signal transduction[65]; they have been linked to inflammation and oxidative stress, and are considered relevant in the context of AD pathology,[66,67] with sex-specific alterations.[62,63] Here, we found a downregulation of LysoPC and LysoPE metabolites in APP KI male mice administered the multi-medications, but not in female mice. Lysophospholipid levels are altered in the brain and periphery during aging and AD,[62,63,67] and its signaling influences microglial activation and inflammatory responses in neurodegenerative diseases.[66] This could correlate with the changes in microglial markers and the positive findings on amyloid pathology. Of interest, in male APP KI mice treated with Combination 1, we found elevated levels of serum kynurenine, which remained unaffected in female mice. The kynurenine pathway as part of tryptophan metabolism appears to be lower in women than men,[68] and dysregulation of the kynurenine signaling is involved in AD pathophysiology.[69] Neuroinflammation can affect kynurenine metabolism in a sex-specific manner, and activation of the kynurenine pathway can be neuroprotective.[70] An upregulation of the kynurenine pathway in males could positively correlate with beneficial cognitive and pathological outcomes. Carnitines were another metabolite class differentially regulated by our treatments. One of its main functions in cellular energy metabolism is fatty acids transport into mitochondria for β-oxidation and energy production. Alteration of these metabolites can lead to impaired brain energy metabolism, negatively affecting cognitive functions.[71] Carnitines are decreased in AD and people with dementia,[72] and increased levels are associated with improved cognitive outcomes.[71,73] Notably, we detected that fatty acids were upregulated in Combination 1–treated APP KI male mice, whereas in treated female mice, both fatty acids and carnitine levels were decreased. These changes might account for the beneficial effects on cognition in male mice, which were absent in female mice. Combination 2 affected far fewer metabolites in APP KI of both sexes compared to Combination 1, indicating a lower impact of this regimen on the metabolome, similar to what was observed in the molecular pathways related to amyloid pathology.

This study presents some limitations. The APP KI mouse model only partially mimics some pathophysiological features of AD pathology, primarily reflecting amyloid pathology and lacking other critical aspects of AD, such as tau pathology and neurodegeneration. This restricts the interpretation of polypharmacy effects to specific pathological features relevant to this particular model. It is also noteworthy to mention that the rapid progression of amyloid load in this mouse model makes it suitable to study specific features of the disease at a young-adult age, still limiting the possibility of studying the treatments in the context of aging. Another important consideration is the variability of the behavioral phenotype in APP KI mice, including the onset of cognitive impairment, observed across our cohorts and in other laboratories.[32,74] This variability may have influenced some treatment outcomes. Considering that amyloid pathology is more pronounced in APP KI female than male mice,[44] this may explain the lack of effects of Combination 1 in female mice, leading to the hypothesis that female mice might need higher doses or earlier treatment than male mice to achieve the same effect. However, monotherapies alone still affected amyloid pathology. Because of feasibility constraints, we did not test all the polypharmacy compounds as monotherapies. We chose three monotherapies based on prior literature studies that indicate a potential role in driving the effects observed with the multi-medication treatments. Finally, this study did not investigate serum levels of drugs and related metabolites; however, they were analyzed in previous polypharmacy mouse models.[75]

5 | CONCLUSIONS

In conclusion, our study showed for the first time sex-specific effects of commonly used multiple-drug therapies (by older adults) in the presence of AD pathology. Our results provide valuable and novel insights into possible molecular targets and serum biomarkers of polypharmacy exposure, which may inform the design of more personalized drug therapies to enhance therapeutic outcomes and minimize adverse effects. The serum metabolomics profiling from our study has clinical implications and provides a solid basis for further investigation of the brain metabolome to identify and validate predictive biomarkers for cognitive and metabolic effects related to polypharmacy. This ultimately offers deeper insights into its impact on both central and peripheral pathways. Our findings suggest that certain combinations could be beneficial in preventing or slowing the progression of ADRD, reinforcing the necessity of understanding sex-specific responses in developing effective treatments. Future research should also focus on elucidating the molecular mechanisms driving the sex differences, particularly in relation to specific pathways affected by polypharmacy, such as inflammation, synaptic dysfunction, and regulation of serotonergic and cholinergic pathways. Favorable combinations will need to be validated in clinical studies for their efficacy and applicability in older adults, to enable the translation of our findings into the clinical setting.

ACKNOWLEDGMENTS

We thank the Animal Behavior Core Facility (ABCF) of Karolinska Institutet, where the behavioral studies were performed, and the Swedish Metabolomic Center (www.swedishmetabolomicscentre.se) for serum metabolomics studies. We also thank Takashi Saito, Takaomi Saido at RIKEN Center for Brain Science and Dr. Per Nilsson for providing the App knock-in mice, Dr. Johanna Wanngren for assistance with breeding, and Felix Andersson for participating in some of the behavioral studies. This work was supported by: Margaretha af Ugglas Foundation, King Gustaf V:s and Queen Victorias Foundation, The Swedish Research Council, The private initiative "Innovative ways to fight Alzheimer´s disease—Leif Lundblad Family and others", Alzheimerfonden, The regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet, Gun och Bertil Stohnes Stiftelse, Stiftelsen Gamla Tjänarinnor, Olle Engkvists Stiftelse.

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest. Author disclosures are available in the supporting information.

REFERENCES

1. Garre-Olmo J. [Epidemiology of Alzheimer's disease and other dementias]. *Rev Neurol*. 2018;66:377-386.
2. Nichols E, Steinmetz JD, Vollset SE, et al. Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019. *Lancet Public Health*. 2022;7:e105-e25.
3. Livingston G, Huntley J, Liu KY, et al. Dementia prevention, intervention, and care: 2024 report of the Lancet standing Commission. *Lancet*. 2024;404:572-628.
4. Pang M, Gabelle A, Saha-Chaudhuri P, et al. Precision medicine analysis of heterogeneity in individual-level treatment response to amyloid beta removal in early Alzheimer's disease. *Alzheimers Dement*. 2024;20:1102-1111.
5. Zhang J, Zhang Y, Wang J, Xia Y, Zhang J, Chen L. Recent advances in Alzheimer's disease: mechanisms, clinical trials and new drug development strategies. *Sig Transduct Target Ther*. 2024;9:211.
6. Santiago JA, Potashkin JA. The impact of disease comorbidities in Alzheimer's disease. *Front Aging Neurosci*. 2021;13:631770.
7. Sharma R, Gill JK, Chhabra M, et al. Prevalence of potentially inappropriate medications in older adults with cognitive impairment or dementia attending memory clinics: a systematic review and meta-analysis. *J Alzheimers Dis*. 2024;101:1107-1120.
8. Morin L, Johnell K, Laroche ML, Fastbom J, Wastesson JW. The epidemiology of polypharmacy in older adults: register-based prospective cohort study. *Clin Epidemiol*. 2018;10:289-298.
9. Strampelli A, Cerreta F, Vučić K. Medication use among older people in Europe: implications for regulatory assessment and co-prescription of new medicines. *Br J Clin Pharmacol*. 2020;86:1912-1920.
10. Wastesson JW, Cedazo Minguez A, Fastbom J, Maioli S, Johnell K. The composition of polypharmacy: a register-based study of Swedes aged 75 years and older. *PLoS One*. 2018;13:e0194892.
11. Cummings J, Zhou Y, Lee G, Zhong K, Fonseca J, Cheng F. Alzheimer's disease drug development pipeline: 2024. *Alzheimer Dement*. 2024;10:e12465.
12. Xu H, Garcia-Ptacek S, Secnik J, et al. Changes in drug prescribing practices are associated with improved outcomes in patients with dementia in Sweden: experience from the Swedish Dementia Registry 2008-2017. *J Am Med Dir Assoc*. 2021;22:1477-1483. e3.
13. Mayburd AL, Koivogui M, Baranova A. Pharmacological signatures of the reduced incidence and the progression of cognitive decline in ageing populations suggest the protective role of beneficial polypharmacy. *PLoS One*. 2019;14:e0224315.
14. Hilmer SN, Gnjidic D. The effects of polypharmacy in older adults. *Clin Pharmacol Ther*. 2009;85:86-88.
15. Katsimpris A, Linseisen J, Meisinger C, Volaklis K. The association between polypharmacy and physical function in older Adults: a systematic review. *J Gen Intern Med*. 2019;34:1865-1873.
16. Koch E, Johnell K, Kauppi K. Longitudinal effects of using and discontinuing central nervous system medications on cognitive functioning. *Pharmacoepidemiol Drug Saf*. 2023;32:446-454.
17. Bonfiglio V, Umegaki H, Kuzuya M. Potentially inappropriate medications and polypharmacy: a study of older people with mild cognitive impairment and mild dementia. *J Alzheimers Dis*. 2019;71:889-897.
18. Thillainadesan J, Gnjidic D, Green S, Hilmer SN. Impact of deprescribing interventions in older hospitalised patients on prescribing and clinical outcomes: a Systematic review of randomised trials. *Drugs Aging*. 2018;35:303-319.
19. Romskaug R, Skovlund E, Straand J, et al. Effect of clinical geriatric assessments and collaborative medication reviews by geriatrician and family physician for improving health-related quality of life in home-dwelling older patients receiving polypharmacy: a cluster randomized clinical trial. *JAMA Intern Med*. 2020;180:181-189.
20. Jing B, Liu X, Graham LA, et al. Deprescribing of Antihypertensive Medications and Cognitive Function in Nursing Home Residents. *JAMA Intern Med*. 2024;184(11):1347-1355.
21. Sawan MJ, Moga DC, Ma MJ, Ng JC, Johnell K, Gnjidic D. The value of deprescribing in older adults with dementia: a narrative review. *Expert Rev Clin Pharmacol*. 2021;14:1367-1382.
22. Eroli F, Johnell K, Latorre-Leal M, et al. Long-term exposure to polypharmacy impairs cognitive functions in young adult female mice. *Aging (Albany NY)*. 2021;13:14729-14744.
23. Eroli F, Johnell K, Latorre Leal M, et al. Chronic polypharmacy impairs explorative behavior and reduces synaptic functions in young adult mice. *Aging (Albany NY)*. 2020;12:10147-10161.
24. Wu H, Mach J, Gemikonakli G, et al. Polypharmacy results in functional impairment in mice: novel insights into age and sex interactions. *J Gerontol A Biol Sci Med Sci*. 2021;76:1748-1756.
25. Hilmer SN, Johnell K, Mach J. Pre-clinical models for geriatric pharmacotherapy. *Drugs Aging*. 2024;41:633-640.
26. Winardi K, Mach J, McKay MJ, et al. Chronic polypharmacy, monotherapy, and deprescribing: understanding complex effects on the hepatic proteome of aging mice. *Aging Cell*. 2025;24:e14357.
27. Hilmer S, Rochon P. Sex and age differences in geriatric pharmacotherapy. *Public Policy Aging Rep*. 2023;33:132-135.
28. Ferretti MT, Iulita MF, Cavedo E, et al. Sex differences in Alzheimer disease — the gateway to precision medicine. *Nat Rev Neurol*. 2018;14:457-469.
29. Zhu D, Montagne A, Zhao Z. Alzheimer's pathogenic mechanisms and underlying sex difference. *Cell Mol Life Sci*. 2021;78:4907-4920.
30. Knufinke M, MacArthur MR, Ewald CY, Mitchell SJ. Sex differences in pharmacological interventions and their effects on lifespan and healthspan outcomes: a systematic review. *Front Aging*. 2023;4:1172789.
31. Saito T, Matsuba Y, Mihira N, et al. Single App knock-in mouse models of Alzheimer's disease. *Nat Neurosci*. 2014;17:661-663.
32. Wang S, Ichinomiya T, Savchenko P, et al. Age-dependent behavioral and metabolic assessment of App NL− G− F/NL− G− F Knock-in (KI) mice. *Front Mol Neurosci*. 2022;15:909989.
33. Wettermark B, Kalantaripour C, Forslund T, Hjemdahl P. Statin treatment for primary and secondary prevention in elderly patients—a cross-sectional study in Stockholm, Sweden. *Eur J Clin Pharmacol*. 2024;80:1571-1580.

34. Petek B, Häbel H, Xu H, et al. Statins and cognitive decline in patients with Alzheimer's and mixed dementia: a longitudinal registry-based cohort study. *Alzheimers Res Ther*. 2023;15:220.
35. Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG. Food intake, water intake, and drinking spout side preference of 28 mouse strains. *Behav Genet*. 2002;32:435-443.
36. Botanas CJ, Bryan de la Peña J, Custodio RJ, et al. Methoxetamine produces rapid and sustained antidepressant effects probably via glutamatergic and serotonergic mechanisms. *Neuropharmacology*. 2017;126:121-127.
37. Latorre-Leal M, Rodriguez-Rodriguez P, Franchini L, et al. CYP46A1-mediated cholesterol turnover induces sex-specific changes in cognition and counteracts memory loss in ovariectomized mice. *Sci Adv*. 2024;10:eadj1354.
38. Pang Z, Lu Y, Zhou G, et al. MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Res*. 2024;52:W398-W406.
39. Lai Y, Liu C-W, Yang Y, Hsiao Y-C, Ru H, Lu K. High-coverage metabolomics uncovers microbiota-driven biochemical landscape of interorgan transport and gut-brain communication in mice. *Nat Commun*. 2021;12:6000.
40. Tu P, Xue J, Niu H, et al. Deciphering gut microbiome responses upon microplastic exposure via integrating metagenomics and activity-based metabolomics. *Metabolites*. 2023;13:530.
41. Pervolaraki E, Hall SP, Foresteire D, et al. Insoluble Aβ overexpression in an App knock-in mouse model alters microstructure and gamma oscillations in the prefrontal cortex, affecting anxiety-related behaviours. *Dis Model Mech*. 2019;12:dmm040550.
42. Kundu P, Torres ERS, Stagaman K, et al. Integrated analysis of behavioral, epigenetic, and gut microbiome analyses in App NL-GF, App NL-F, and wild type mice. *Sci Rep*. 2021;11:4678.
43. Mehla J, Lacoursiere SG, Lapointe V, et al. Age-dependent behavioral and biochemical characterization of single APP knock-in mouse (APPNL-GF/NL-GF) model of Alzheimer's disease. *Neurobiol Aging*. 2019;75:25-37.
44. Masuda A, Kobayashi Y, Kogo N, et al. Cognitive deficits in single App knock-in mouse models. *Neurobiol Learn Mem*. 2016;135:73-82.
45. Mach J, Gemikonakli G, Logan C, et al. Chronic polypharmacy with increasing drug burden index exacerbates frailty and impairs physical function, with effects attenuated by deprescribing, in aged mice. *J Gerontol A Biol Sci Med Sci*. 2021;76:1010-1018.
46. LeGates TA, Kvarta MD, Thompson SM. Sex differences in antidepressant efficacy. *Neuropsychopharmacology*. 2019;44:140-154.
47. Zhang L, Ma W, Barker JL, Rubinow DR. Sex differences in expression of serotonin receptors (subtypes 1A and 2A) in rat brain: a possible role of testosterone. *Neuroscience*. 1999;94:251-259.
48. Ladage D, Schwinger RH, Brixius K. Cardio-selective beta-blocker: pharmacological evidence and their influence on exercise capacity. *Cardiovasc Ther*. 2013;31:76-83.
49. Tran T, Mach J, Gemikonakli G, et al. Diurnal effects of polypharmacy with high drug burden index on physical activities over 23 h differ with age and sex. *Sci Rep*. 2022;12:2168.
50. Balthazar L, Lages Y, Romano V, Landeira-Fernandez J, Krahe T. The association between the renin-angiotensin system and the hypothalamic-pituitary-adrenal axis in anxiety disorders: a systematic review of animal studies. *Psychoneuroendocrinology*. 2021;132:105354.
51. Zhang S, He L. Captopril reverses chronic unpredictable mild stress-induced depression-like behavior in rats via bradykinin-B2r signaling pathway. *Trop J Pharma Res*. 2022;21:2131-2137.
52. Fischer M, Baessler A, Schunkert H. Renin angiotensin system and gender differences in the cardiovascular system. *Cardiovasc Res*. 2002;53:672-677.
53. Ueno K, Sato H. Sex-related differences in pharmacokinetics and pharmacodynamics of anti-hypertensive drugs. *Hypertens Res*. 2012;35:245-250.
54. Chandra S, Jana M, Pahan K. Aspirin induces lysosomal biogenesis and attenuates amyloid plaque pathology in a mouse model of Alzheimer's disease via PPARα. *J Neurosci*. 2018;38:6682-6699.
55. Weng J, Zhao G, Weng L, Guan J, Aspirin using was associated with slower cognitive decline in patients with Alzheimer's disease. *PLoS One*. 2021;16:e0252969.
56. Tong X-K, Royea J, Hamel E. Simvastatin rescues memory and granule cell maturation through the Wnt/β-catenin signaling pathway in a mouse model of Alzheimer's disease. *Cell Death Dis*. 2022;13:325.
57. Schilling JM, Cui W, Godoy JC, et al. Long-term atorvastatin treatment leads to alterations in behavior, cognition, and hippocampal biochemistry. *Behav Brain Res*. 2014;267:6-11.
58. Beaman EE, Bonde AN, Larsen SMU, et al. Blood–brain barrier permeable β-blockers linked to lower risk of Alzheimer's disease in hypertension. *Brain*. 2022;146:1141-1151.
59. Evans AK, Ardestani PM, Yi B, Park HH, Lam RK, Shamloo M. Beta-adrenergic receptor antagonism is proinflammatory and exacerbates neuroinflammation in a mouse model of Alzheimer's Disease. *Neurobiol Dis*. 2020;146:105089.
60. Reddy AP, Sawant N, Morton H, et al. Selective serotonin reuptake inhibitor citalopram ameliorates cognitive decline and protects against amyloid beta-induced mitochondrial dynamics, biogenesis, autophagy, mitophagy and synaptic toxicities in a mouse model of Alzheimer's disease. *Hum Mol Genet*. 2021;30:789-810.
61. Sheline YI, West T, Yarasheski K, et al. An antidepressant decreases CSF Aβ production in healthy individuals and in transgenic AD mice. *Sci Transl Med*. 2014;6:236re4-re4.
62. Strefeler A, Jan M, Quadroni M, et al. Molecular insights into sex-specific metabolic alterations in Alzheimer's mouse brain using multi-omics approach. *Alzheimers Res Ther*.. 2023;15:8.
63. Pandey RS, Arnold M, Batra R, et al. Metabolomics profiling reveals distinct, sex-specific signatures in serum and brain metabolomes in mouse models of Alzheimer's disease. *Alzheimer Dement*. 2024;20:3987-4001.
64. Arnold M, Nho K, Kueider-Paisley A, et al. Sex and APOE ε4 genotype modify the Alzheimer's disease serum metabolome. *Nat Commun*. 2020;11:1148.
65. Farooqui AA, Horrocks LA, Farooqui T. Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. *Chem Phys Lipids*. 2000;106:1-29.
66. Gaire BP, Choi J-W. Critical roles of lysophospholipid receptors in activation of neuroglia and their neuroinflammatory responses. *Int J Mol Sci*. 2021;22:7864.
67. Peña-Bautista C, Roca M, López-Cuevas R, Baquero M, Vento M, Chafer-Pericas C. Metabolomics study to identify plasma biomarkers in Alzheimer disease: apoE genotype effect. *J Pharm Biomed Anal*. 2019;180:113088.
68. Theofylaktopoulou D, Midttun Ø, Ulvik A, et al. A community-based study on determinants of circulating markers of cellular immune activation and kynurenines: the Hordaland Health Study. *Clin Exp Immunol*. 2013;173:121-130.
69. Liang Y, Xie S, He Y, et al. Kynurenine pathway metabolites as biomarkers in Alzheimer's disease. *Dis Markers*. 2022;2022:9484217.
70. Knapskog AB, Aksnes M, Edwin TH, et al. Higher concentrations of kynurenic acid in CSF are associated with the slower clinical progression of Alzheimer's disease. *Alzheimer Dement*. 2023;19:5573-5582.
71. Owen L, Sunram-Lea SI. Metabolic agents that enhance ATP can improve cognitive functioning: a review of the evidence for glucose, oxygen, pyruvate, creatine, and L-carnitine. *Nutrients*. 2011;3:735-755.

72. Cristofano A, Sapere N, La Marca G, et al. Serum levels of acyl-carnitines along the continuum from normal to Alzheimer's dementia. *PLoS One*. 2016;11:e0155694.
73. Kepka A, Ochocinska A, Borzym-Kluczyk M, et al. Preventive role of L-carnitine and balanced diet in Alzheimer's disease. *Nutrients*. 2020;12:1987.
74. Sakakibara Y, Sekiya M, Saito T, Saido TC, Iijima KM. Amyloid-β plaque formation and reactive gliosis are required for induction of cognitive deficits in App knock-in mouse models of Alzheimer's disease. *BMC neuroscience*. 2019;20:1-14.
75. Mach J, Wang X, Hilmer SN. Quantification of serum levels in mice of seven drugs (and six metabolites) commonly taken by older people with polypharmacy. *Fundam Clin Pharmacol*. 2021;35:410-422.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Eroli F, Johnell K, Acararicin Z, et al. Commonly prescribed multi-medication therapies exert sex-specific effects on Alzheimer's disease pathology and metabolomic profiles in $App^{NL\text{-}G\text{-}F}$ mice: Implications for personalized therapeutics in aging. *Alzheimer's Dement*. 2025;21:e70081. https://doi.org/10.1002/alz.70081