University of Crete

School of Medicine

Graduate Program in Neuroscience

Neurogenesis and Neurodegeneration group

Netherlands Institute for Neuroscience

Master Thesis

Deciphering molecular regulators of neuroinflammation in Alzheimer's disease using iPSC-derived microglia

//

Αποκωδικοποιώντας τους μοριακούς ρυθμιστές της νευροφλεγμονής στη νόσο Αλτσχάιμερ με τη χρήση επαγόμενων πολυδύναμων βλαστικών κυττάρων μικρογλοίας

Anita Lygeroudi

Supervisor: Dr. Evgenia Salta

Amsterdam
December 2022 – August 2023

Date of submission: 24/08/2023

BODOSSAKI FOUNDATION

NETHERLANDS INSTITUTE FOR NEUROSCIENCE

Evaluation Committee

Amber Penning, Evgenia Salta, Sarah Snoeck

Netherlands Institute for Neuroscience

Dr. Evgenia Salta

Group Leader

Amber Penning

PhD student

Sarah Snoeck

Lab technician

Acknowledgements

I would first like to express my sincere thanks to Dr Evgenia Salta, whose assistance and expertise throughout the process was invaluable.

I would also like to express my very great appreciation to my daily supervisor, Amber Penning, for her support and inspiring mentorship that has been truly instrumental in the success of this project.

Additionally, I want to express my gratitude to Sarah Snoeck for always providing me with valuable guidance and technical support during the experimental stages of this project.

Furthermore, I thank Dilara Ayyildiz, Giorgia Tosoni and Oliver Polzer for their insightful feedback and stimulating discussions.

I would also like to thank Carla Rodriguez Mogeda for her assistance and valuable suggestions in the data analysis.

Lastly, I extend my heartfelt appreciation to my friends and family for always supporting me and encouraging me to follow my dreams.

Without all of your support this thesis would have never been accomplished.

Curriculum Vitae

Anita Lygeroudi

Postgraduate Student in Neuroscience

Date of Birth: 26/01/1997
Mobile: +306980972939
Mail: anita.lyger@gmail.com
Address: Skylitsi 45-47, Piraeus 18533, Greece
LinkedIn Profile URL: linkedin.com/in/anita-lygeroudi-12a686198

Soft Skills

Adaptability
Resourcefulness
Creative and Team Worker
Emotional Quality

Laboratory Skills

Behavioral experiments
Tissue Clearing
Whole blood cell isolation
Bisulphite treatment
PCR & qPCR
Pyrosequencing
Cancer cell culture
DNA/RNA isolation
Immunohistochemistry
Immunocytochemistry
Confocal Imaging

Languages

Greek (Native)
English (Cambridge CPE)

Computer skills

MS Office (Word, Excel, Power Point)

Hobbies/ Interests

Travelling
PoEtry
PoTery
Exercise
Food
Crafting

Profile *An enthusiastic and highly motivated individual that is always searching for opportunities that will help them unlock their full potential*

Education

University of Crete — Crete, Greece
MSc in Neurosciences — 2021 –

Democritus University of Thrace — Alexandroupolis, Greece
BSc in Molecular Biology and Genetics — 2015 – 2019

Ionidios Model School of Piraeus — Piraeus, Greece
Graduated with Honours — 2012 – 2015

Work & Research Experience

Netherlands Institute for Neuroscience — Amsterdam, The Netherlands
Neurogenesis & Neurodegeneration Group — Dec – Aug 2023
Netherlands Institute for Neuroscience (NIN)
MSc Thesis "Deciphering molecular regulators of Neurogenesis & Neuroinflammation in Alzheimer's disease using iPSC-derived neurons & microglia"
Group Leader: Dr Evgenia Salta

Central Laboratories — Athens, Greece
Clinical Lab Molecular Biologist — 2020 – 2021
Molecular Diagnostics Department
In Vitro diagnosis of SARS-CoV2, HBV, HCV, CFTR, HLA β*27, Thrombophilias, Influenza A & B
Skill development: Sample collection, Result evaluation, Client facilitation, Colleague training and collaboration

University of Liverpool — Liverpool, UK
Respiratory Cancer Biomarkers Group — Mar – July 2019
Department of Molecular & Clinical Cancer Medicine
Institute of Translational Medicine
B.A Thesis "Sortilin 1B Methylation as a Potential Diagnostic Biomarker in Lung Cancer"
Supervisor: Dr Triantafillos Liloglou

Biomedical Research Foundation of the Academy of Athens — Athens, Greece
Internship, Research in Translational Neurobiology — June – July 2018
Familiarised myself with molecular techniques and research concepts
Supervisor: Dr Leonidas Stefanis

Honours & Awards

Bodossaki Foundation Scholarship — Crete, Greece
Received the Bodossaki Foundation Scholarship to complete my MSc program in Neurosciences at the University of Crete — 2022-2023

Erasmus+ Scholarship — Crete, Greece
Participated in the Erasmus+ Exchange Program to conduct my MSc Thesis at the Netherlands Institute for Neuroscience — Dec 2022 – Aug 2023

Erasmus+ Scholarship — Alexandroupolis, Greece
Participated in the Erasmus+ Exchange Program to conduct my B.A Thesis at the University of Liverpool — Mar 2019 – July 2019

Conferences

Dutch Neuroscience Meeting — Tiel, Netherlands
Poster presentation — June, 2023

Student-Run-Biology-Workshop — Alexandroupolis, Greece
Team Facilitator — 2017 – 2018
Organiser — 2016 – 2017
Participant — 2015 – 2016

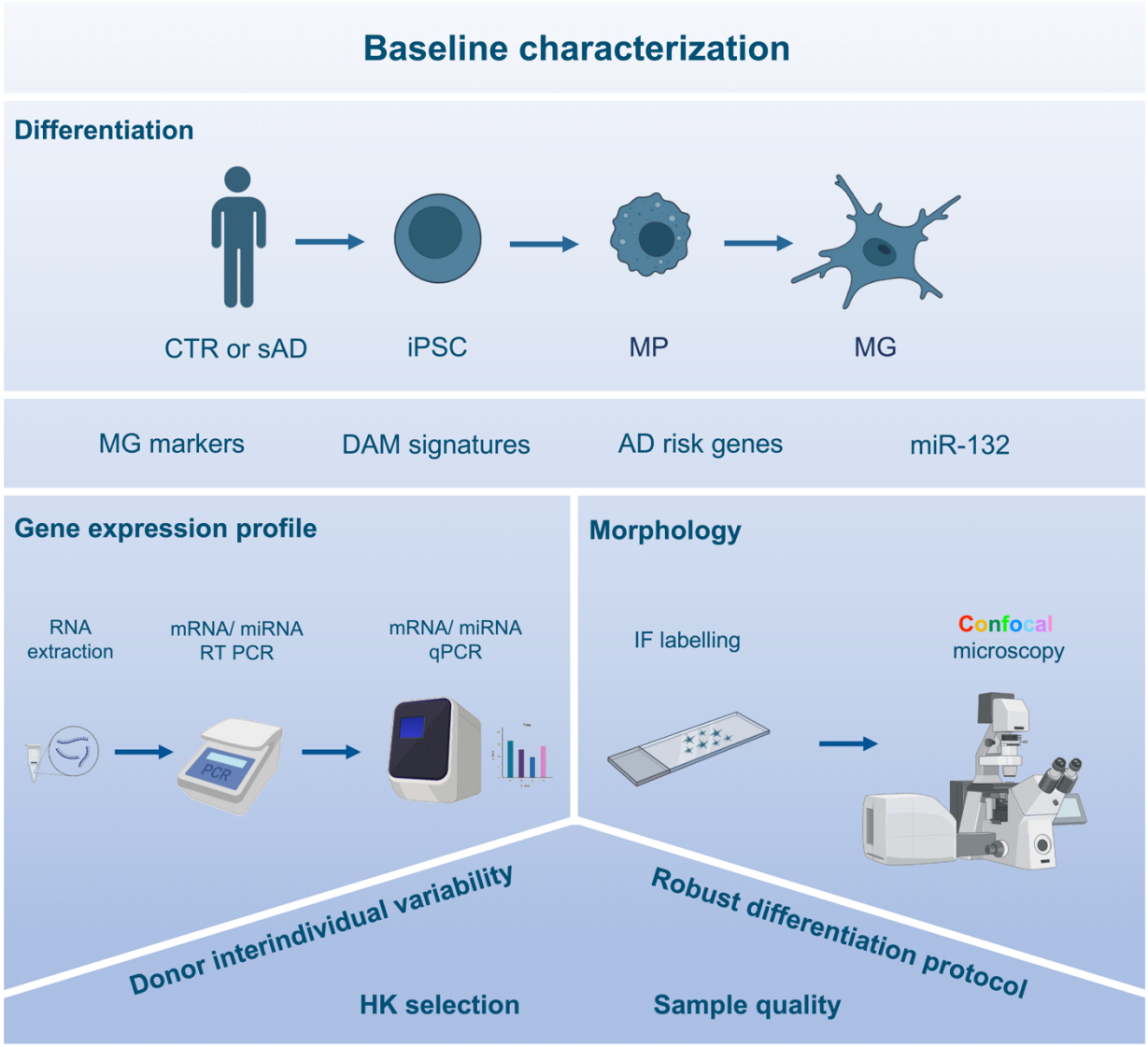
Abstract (English)

Alzheimer's disease (AD) is a fatal neurodegenerative disease with no available treatment. Since the generation of induced pluripotent stem cells (iPSCs), several cell-based models of different diseases have been developed. In an attempt to better understand the disease aetiology and identify effective therapeutics for AD, human iPSC lines have been utilised to study both the familial (fAD) and the sporadic (sAD) form of AD. However, there has not yet been a systematic and detailed characterization of microglia derived from human sAD iPSC lines at baseline. By monitoring gene expression profiles using quantitative PCR and immunofluorescent labelling, we focus on the characterization of two iPSC lines derived from healthy individuals and one iPSC line derived from a patient with sAD. We found that iPSC-derived microglia highly express microglial markers, thereby validating their cellular identity in our experimental setting. In addition, we showed an upregulation of DAM and AD risk genes in the sAD iPSC line, and we observed distinct morphological characteristics in the sAD iPSC line compared to controls, suggestive of a more activated phenotype, recapitulating microglial behavior in AD brain. Interestingly, despite these observations no downregulation of miR-132 expression levels in the sAD iPSC line was observed. This study will provide fundamental knowledge for future reference, to better evaluate prospective results under different treatment conditions.

Abstract (Greek)

Η νόσος Αλτσχάιμερ (AD) είναι μια θανατηφόρα νευροεκφυλιστική νόσος, χωρίς διαθέσιμη αποτελεσματική θεραπεία. Η εξέλιξη της τεχνολογίας των επαγόμενων πολυδύναμων βλαστικών κυττάρων (iPSCs), οδήγησε στην ανάπτυξή μιας πληθώρας κυτταρικών μοντέλων για τη μελέτη ποικίλων νόσων. Με απώτερο σκοπό την διερεύνηση των μοριακών μηχανισμών λειτουργίας της νόσου Αλτσχάιμερ, καθώς και την ανάπτυξη αποτελεσματικής θεραπείας, έχουν χρησιμοποιηθεί ανθρώπινες iPSC σειρές για τη μελέτη, τόσο της οικογενούς (fAD) όσο και της σποραδικής (sAD) μορφής της νόσου. Ωστόσο, απουσιάζει από τη βιβλιογραφία ένας λεπτομερής και συστηματικός χαρακτηρισμός των μικρογλοιακών κυττάρων από ανθρώπινες sAD iPSC σειρές. Ανιχνεύοντας τα επίπεδα έκφρασης, χρησιμοποιώντας ποσοτική PCR (qPCR) και ανοσοφθορισμό, η παρούσα μελέτη εστιάζει στον χαρακτηρισμό δυο iPSC σειρών από υγιή άτομα και μιας iPSC σειράς από έναν ασθενή με sAD. Συγκεκριμένα, παρατηρήθηκε υψηλή έκφραση μικρογλοιακών δεικτών από τα διαφοροποιημένα iPS μικρογλοιακά κύτταρα, επιβεβαιώνοντας την κυτταρική τους ταυτότητα. Επιπλέον, ανιχνεύθηκε μία αύξηση της έκφρασης των γονιδίων DAM και του APOE, του σημαντικότερου δείκτη κινδύνου στη νόσο Αλτσχάιμερ, στη sAD iPSC σειρά, υποδηλώνοντας έναν πιο ενεργοποιημένο φαινότυπο, ο οποίος είναι δυνατό να αναπαραχθεί in vitro. Ταυτόχρονα, ιδιαίτερο ενδιαφέρον αποτελεί το γεγονός ότι δεν παρατηρήθηκε μείωση της έκφρασης των επιπέδων του miR-132 στη sAD iPSC σειρά. Η συγκεκριμένη μελέτη αποσκοπεί στην παροχή θεμελιωδών γνώσεων, χρήσιμων στο πλαίσιο μελλοντικής αναφοράς, με στόχο τη βελτιστοποίηση της αξιολόγησης προσεχών αποτελεσμάτων, υπό όμοιων αλλά και διαφορετικών συνθηκών έρευνας.

Graphical abstract



Keywords

Alzheimer's disease; induced pluripotent stem-cells; sporadic Alzheimer's disease; microglia; baseline characterisation

Table of Contents

Abbreviations

AD: Alzheimer's disease

NFTs: Neurofibrillary tangles

fAD: Familial Alzheimer's disease

sAD: Sporadic Alzheimer's disease

CNS: Central nervous system

APP: Amyloid precursor protein

PSEN1: Presenilin 1

PSEN2: Presenilin 2

iPSCs: Induced Pluripotent Stem Cells

ES: Embryonic stem cells

Aβ: Amyloid-beta

MGL(s): Microglia-like cell(s)

TREM2: Triggering receptor expressed on myeloid cells 2

2D: Two-dimensional

3D: Three-dimensional

GWAS: Genome-wide association studies

miRNA: microRNA

miR-132: microRNA-132

CHAPTER 1. Introduction

Alzheimer's disease (AD) is a devastating progressive neurodegenerative disorder and currently the most common form of dementia, accounting for 60-70% of all dementia cases (World Health Organization, 2023). Early-onset familial AD (fAD) usually occurs between the third and fifth decade of life, whilst the sporadic late-onset AD (sAD), which represents the vast majority of AD cases (~95%), often develops after the age of 65 (Gaugler et al., 2016; Lane et al., 2018; Long & Holtzman, 2019). AD is characterized by two neuropathological hallmarks, shared by both fAD and sAD, which are the abnormal deposition of amyloid-β peptides (Aβ) into extracellular plaques and the aggregation of hyperphosphorylated TAU into intraneuronal neurofibrillary tangles (NFTs) (Jeong, 2017; Long & Holtzman, 2019; Reiman et al., 2020). The amyloid cascade hypothesis postulates that Aβ plaques gradually lead to TAU pathology, and consequently to synaptic dysfunction and neuronal death, resulting in the observed cognitive and functional decline of AD patients. However, the sequential nature of this cascade is still a matter of debate within the research community (De Strooper & Karran, 2016). While it is well understood that fAD occurs from mutations in genes such as the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*), sAD etiology is much more complicated. Several risk factors such as age, sex, genetic background, vascular diseases as well as environmental factors are found to play important roles in disease development and progression, rendering sAD a multifactorial disorder (Andrews et al., 2023; Talwar et al., 2016). Nonetheless, in both fAD and sAD, there is increasing evidence suggesting that the manifestation of the disease is most likely due to intricate interactions between various types of brain cells that lead to interrelated cellular pathologies (De Strooper & Karran, 2016).

During recent years, our understanding of the pathomechanisms that underlie AD has significantly advanced. However, all of the efforts that have been made to translate this knowledge into effective treatments for AD have floundered (Cummings, 2018). This has been attributed to a multitude of factors, including concerns around the fAD animal models that are employed during the early stages of drug development (Laurijssens et al., 2013). Relevant to research that has utilised stem cell technologies, a significant contributing factor to the unsuccessful attempts towards an effective treatment for AD, is the fact that most of these studies have employed induced pluripotent stem cells (iPSCs) from patients with fAD, despite the higher prevalence and complexity associated with sAD aetiology (Riemens et al., 2020). Consequently, while these studies have provided us with valuable insights, they have failed to

address the entire spectrum of sAD pathology, which could account for the challenges that the research community has encountered in the development of therapeutic treatments for AD.

Accumulating evidence has pointed to the pivotal role of neuroinflammation in the pathogenesis of AD, mediated primarily by microglia, the innate immune cells of the central nervous system (Leng & Edison, 2021). Both animal and human studies have previously reported microglial activation in the early stages of AD and although neuroinflammation can have neuroprotective properties, prolonged neuroinflammation can be detrimental and has been associated with neurodegeneration (Hansen et al., 2018; Hanzel et al., 2014; Hickman et al., 2018; Okello et al., 2009). Thus, promoting the beneficial aspects of neuroinflammation, while dampening the detrimental responses holds great therapeutic potential for battling AD.

As mentioned, microglia are the innate immune cells of the central nervous system (CNS). Early in development microglia originate from the yolk sac as MYB-independent macrophages, where they migrate into the developing brain to complete differentiation (Ginhoux et al., 2010; Kierdorf et al., 2013; Schulz et al., 2012). Microglia play essential roles in the CNS, as phagocytes that constantly survey their environment. They are involved in neurogenesis, synaptic plasticity, immune responses against CNS injury and pathology, as well as homeostatic regulation and tissue maintenance (Borst et al., 2021; Colonna & Butovsky, 2017). While their implication in neurodegenerative diseases has long been recognized, it is only recently that their active role as a contributing factor to Alzheimer's disease has been fully appreciated (Heppner et al., 2015; Young-Pearse et al., 2023). Several genes related to microglial function have been identified as risk factors for developing sAD in genome-wide association studies (GWAS), including *TREM2*, *CD33*, *SPI1*, and *MS4A6A*, yet their functional roles and implications to the pathogenesis of sAD remain largely unknown (Hansen et al., 2018; Huang et al., 2017; Jonsson et al., 2013; Lambert et al., 2013, 2023; Sims et al., 2017; Villegas-Llerena et al., 2016). Thus, studying microglia in a neurodegenerative environment, like sAD, will improve our understanding of the development of the disease. However, in order to do that, reliable *in vitro* models need to be developed, that will authentically mimic human microglia *in vivo*.

Most of the research that has been conducted to study microglia thus far, has utilised rodent models and it has proven to be invaluable when it comes to understanding the origin and functionality of microglia in vivo. Nonetheless, it is widely appreciated that there are interspecies differences between human and rodent microglia, such as in volume, density and expression profiles that render these models ultimately inadequate, when attempting to

comprehend the whole spectrum of human microglial functions in vivo (Schwabenland et al., 2021; Yvanka de Soysa et al., 2022). When looking deeper into the protein sequence of specific sAD risk genes, including microglial cell surface receptor genes (e.g., *TREM2*, *CD33*, *MS4A6A*), only 50% of the protein sequence is identical between mouse and humans. In addition, human microglia exhibit notable differences in their activation patterns compared to murine microglia (Friedman et al., 2018; Geirsdottir et al., 2019; Gosselin et al., 2017; Hasselmann & Blurton-Jones, 2020; Mancuso et al., 2019; Zerbino et al., 2018). Distinct states of mouse microglia have been identified, that are temporally and spatially regulated, based on their expression profile (Masuda et al., 2019; Mathys et al., 2017), while human microglial states are far more complicated due to their functional heterogeneity and their classification is much more challenging (Chen & Colonna, 2021; Sankowski et al., 2019). This, along with the fact that numerous genes that are regulated by aging mechanisms exhibit limited overlap in mouse and human microglia, underscores the disparities between the two species and makes the translatability of the results even more daunting, highlighting the importance of studying human iPSC-derived microglia-like cells (MGLs) to understand the pathomechanisms involved in AD (Galatro et al., 2017; Grabert et al., 2016).

The advent of single-cell technologies has revolutionized our understanding of the diverse human microglial states in vivo, nevertheless the research community is just starting to comprehend all of the aspects of these multifaceted cells (Chen & Colonna, 2021; Hansen et al., 2018). One microglial state that has attracted a lot of interest due to its expression of many AD risk genes, such as *APOE* and *TREM2*, is the disease-associated microglia state (DAM) (Hansen et al., 2018; Keren-Shaul et al., 2017). DAMs are characterized by the upregulation of genes associated with immune responses and pro-inflammatory processes and downregulating genes related to homeostatic functions (Keren-Shaul et al., 2017; Orre et al., 2014; Srinivasan et al., 2016; Wang et al., 2016). There is evidence suggesting that this state might have a beneficial impact on attenuating the spread of Aβ (Deczkowska et al., 2018; Keren-Shaul et al., 2017), but the exact role of DAMs in AD remains to be elucidated.

Protocols to differentiate microglia from human iPSCs have only recently become available. Previous efforts to create MGLs had been made, but none had focused specifically on generating microglia from human iPSCs due to challenges associated with their unique developmental origin (Mungenast et al., 2016). Since the development of these protocols, a plethora of studies have been conducted to gain a comprehensive understanding of the properties of these cells under both physiological and pathological conditions. Collectively,

monocultured MGLs appear to resemble human primary microglia in terms of their morphology, expression profile and functionality (Abud et al., 2017; Brownjohn et al., 2018; Douvaras et al., 2017; Dräger et al., 2022; Haenseler et al., 2017). Under physiological conditions, microglia-like cells have a ramified morphology and express cell surface microglia markers such us IBA1, TMEM119 or P2RY12 (Abud et al., 2017; Douvaras et al., 2017; Haenseler et al., 2017; Muffat et al., 2016; Pandya et al., 2017). On a whole-transcriptome level, the expression profile of MGLs bear similarities with that of human primary microglia and in terms of their function they secrete a battery of cytokines upon stimulation, they demonstrate phagocytic abilities and release detectable calcium transients in response to ADP (Abud et al., 2017; Brownjohn et al., 2018; Douvaras et al., 2017; Haenseler et al., 2017). Under pathological conditions, such as Alzheimer's disease, the major risk factor *APOE4* has been demonstrated to have an influence on various iPSC-derived MGLs phenotypes with *APOE4* MGLs displaying a pro-inflammatory gene expression profile and impaired Aβ phagocytosis (Lin et al., 2018; Moser et al., 2021; Reich et al., 2021; TCW et al., 2022).

Apart from human iPSC-derived microglia monoculture protocols, a range of methodologies to investigate microglia have been developed in these past few years. These approaches encompass mouse models, iPSC-derived microglia co-cultures in both two-dimensional (2D) and three-dimensional (3D) settings, as well as the utilization of xenograft transplantations and postmortem human brain tissue samples. Each of these methodologies has provided valuable insights into the complexities of microglial function and how the existing protocols can be improved to achieve more accurate and reliable results.

Xenotransplantation models in which MGLs were transplanted into the brain of mouse models, showed that the resulting MGLs had a more mature and functional phenotype that better resembled microglia in vivo (Gosselin et al., 2017; Hasselmann & Blurton-Jones, 2020; Mancuso et al., 2019; Schafer et al., 2023; Xu et al., 2020). In another study, MGLs co-cultured with neurons in a 2D setting, were found to have altered expression profiles of microglial genes, suggesting that the presence of specific factors in the co-culture environment is essential to drive microglia towards their maturation (Abud et al., 2017). In a subsequent study using a 3D culture system where MGLs were co-cultured with neurons and astrocytes, Lin et al. demonstrated that MGLs can be successfully integrated into cerebral organoids and that their morphology differs from MGLs cultured in a 2D environment (Lin et al., 2018). From studies that have integrated data from human post-mortem samples into their analyses, it has been shown that the culture environment can drastically affect the transcription profile of human

primary microglia when cultured. This raises concerns regarding the use of cultured human primary microglia and given their complexity and dynamic nature, it is preferable for studies that analyse human brain samples to directly process them rather than culturing and maintaining the microglia in vitro (Brownjohn et al., 2018; Butovsky et al., 2014).

From the aforementioned, it is apparent that protocols for co-culturing MGLs either in 2D or 3D culture systems will be instrumental for studying the dynamic interactions between different brain cell types in the future. However, since MGLs have been reported to express genes associated with neurodegenerative diseases, including AD, monocultures can be useful for reductionist studies that focus on answering specific disease-related questions (Brownjohn et al., 2018; Haenseler et al., 2017). This highlights the necessity of generating iPSC-derived MGLs that accurately mimic in vivo microglia in an in vitro environment, where their manipulation is possible.

The continuous refinement of these protocols with the aim of better mimicking the in vivo conditions, will enhance our ability to model more authentically Alzheimer's disease using iPSCs. Despite the progress that has been made in the field, we still have a lot to explore in regard to the functionality of the genes that have been associated with an increased risk for developing sAD in distinct brain cell types, the potential implications of these mutations on the crosstalk amongst the different brain cells and how the regulation of putative therapeutic targets might influence their function.

miRNAs have emerged as potent regulators of a plead of AD-related processes in a wide range of brain cell populations (Bhatnagar et al., 2023; Walgrave, Zhou, et al., 2021). miR-132 has been reported to be the most consistently and robustly downregulated miRNA in AD (Lau et al., 2013) and has emerged as a promising therapeutic target for battling AD pathology, as it can ameliorate key endophenotypes, including memory deficits (El Fatimy et al., 2018; Salta & De Strooper, 2017, PMID: 34033742; Walgrave, Balusu, et al., 2021). However, before proceeding on manipulating the expression levels of miR-132, it is imperative that we first establish a baseline understanding of the properties of microglia in human sAD iPSC lines, since there has not yet been a systematic and detailed characterisation of these cells under this specific context. Here, by monitoring gene expression profiles using quantitative PCR and immunofluorescent labelling, we focus on the characterisation of three iPSC lines derived from two healthy individuals and one iPSC line derived from a patient with sAD. We find that iPSC-derived microglia highly express mature microglial markers. In addition, we show an upregulation of

DAM and AD risk genes in the sAD iPSC line, and we observed distinct morphological characteristics in the sAD iPSC line compared to controls. Interestingly, no downregulation of miR-132 expression levels in the sAD iPSC line was observed. This study will provide fundamental knowledge for future reference, to better evaluate prospective results under different treatment conditions

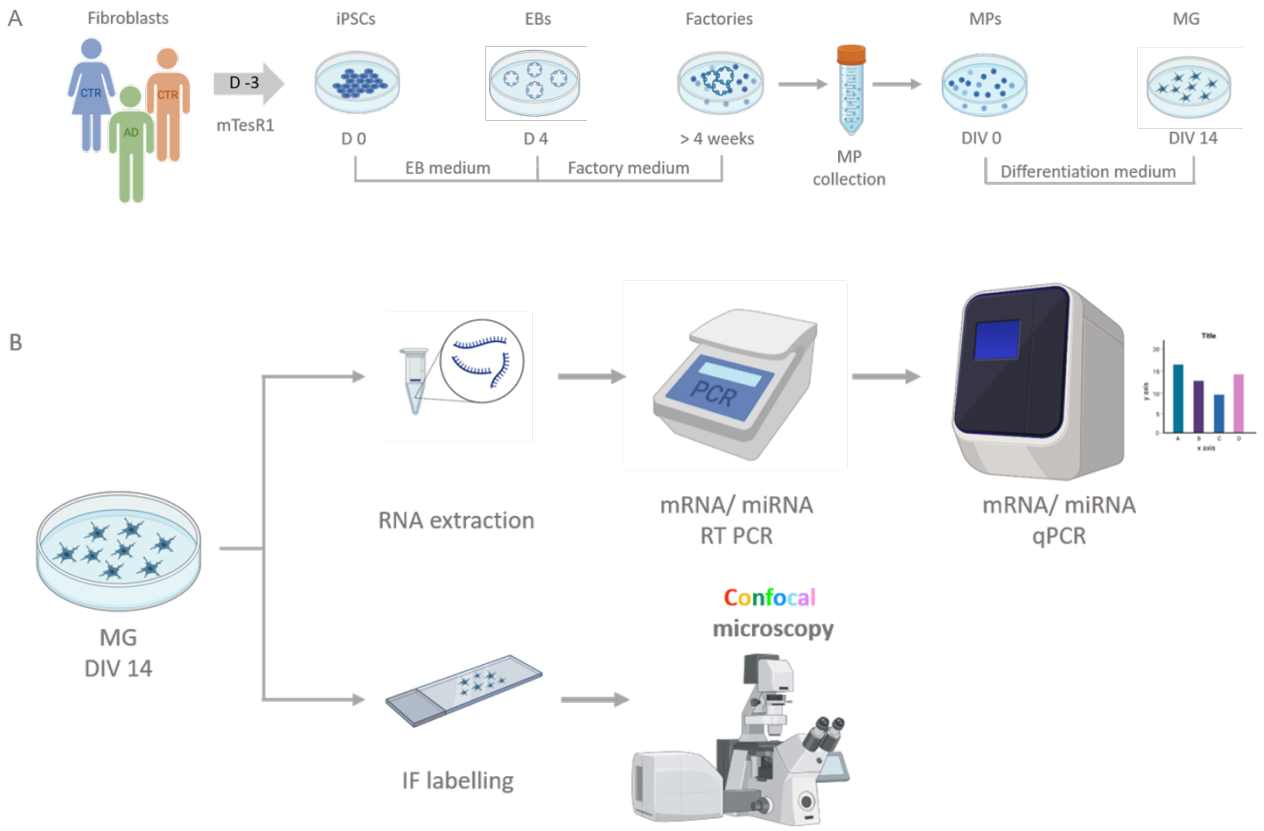


Figure 1. **Workflow for the characterisation of human iPSC-derived microglia.**
A. Schematic representation of the differentiation protocol of human iPSC-derived microglia. CTR, control; AD, Alzheimer's disease; iPSCs, induced pluripotent stem cells; EBs, embryoid bodies; MPs, macrophage precursors; MG, microglia; DIV, days in vitro. **B**. Methods employed for the characterisation of human iPSC-derived microglia. RT PCR, reverse transcriptase PCR; qPCR, quantitative PCR; IF, immunofluorescence.

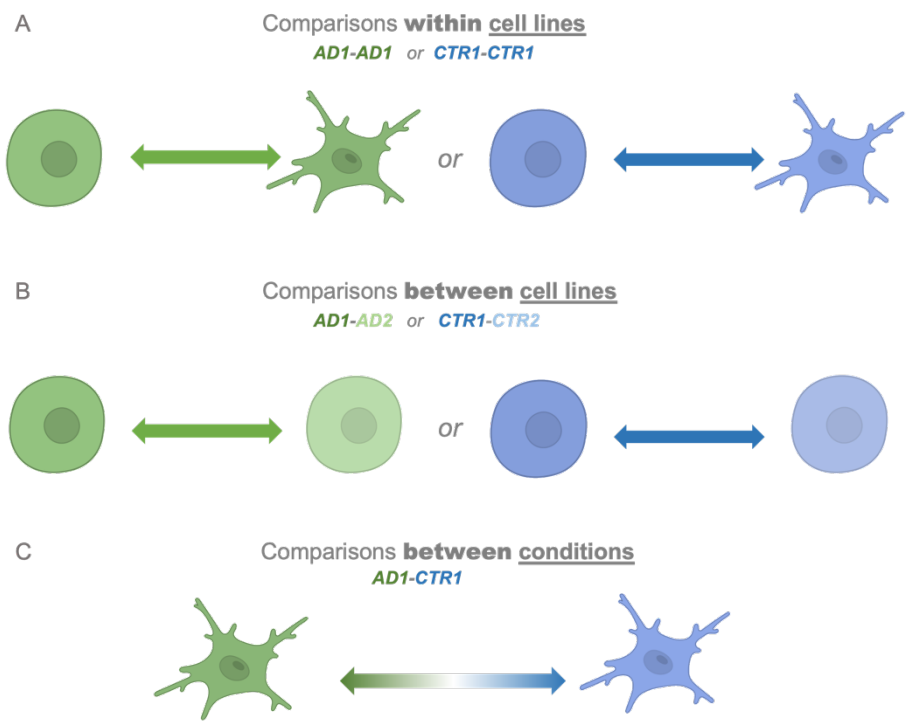


Figure 2. **Schematic representation of the conducted comparisons.**
For the characterization of the differentiated microglia a series of comparisons were made within the cell lines (A), between the cell lines (B) and between the different conditions (C).

CHAPTER 2. Methodology

2.1 Microglial differentiation from human induced pluripotent stem cells (iPSCs)

Human induced pluripotent stem cells (iPSCs) (Coriell iPSCs ID; Ctr AG27611, Ctr AG27602 and sAD AG27605, Table 1) were utilized for the generation of microglia following a previously described protocol (Haenseler et al., 2017). iPSCs were rapidly thawed in a 6-well plate coated with matrigel (Corning® Matrigel® hESC-Qualified, Cat#354277) and cultured in mTeSR1 (Cat#85850, Stemcell), supplemented with 10 µM Y-27632 (Rock inhibitor, Cat#72304, Stemcell) for the initial 24 hours (Figure 1A). Subsequently, when iPSCs reached confluency, they were transferred to an AggreWell 800 (Cat#34815, Stemcell) for Embryoid Body (EB) formation at a final concentration of 4×10^6 cells per well. The cells were maintained in mTeSR1 medium supplemented with 50 ng/mL BMP4 (Cat#PHC9531, Thermo Fisher Scientific), 50 ng/mL VEGF (Cat#100-20, Peprotech), and 20 ng/mL SCF (Cat#130-096-693, Miltenyi Biotec) for three days. The plate was kept in 37°C, under 5% CO2 and the medium was refreshed every 24 hours. On day four, the EBs were transferred to 6-well plates (approximately 15 EBs/well) in X-VIVO15 (Cat#BE02-060F, Lonza), supplemented with 2 mM Glutamax (Cat#35050038, Life Technologies), 50 µM β-mercaptoethanol (Cat#31350010, Thermo Fisher Scientific), 50 U/ml penicillin/streptomycin (Cat#P4333-100ml, Sigma-

Aldrich), 25 ng/ml Il-3 (Cat#PHC0035, Thermo Fisher Scientific), and 100 ng/mL M-CSF (Cat#PHC9501, Thermo Fisher Scientific), with fresh factory medium added every week. Macrophage precursors were harvested from eight-week-old factories and filtered with a 40 µm cell strainer. Next, the macrophage precursors were centrifuged at 400 xg for 5 min and re-suspended in differentiation medium for differentiation into microglia. Differentiation medium consisted of Advanced DMEM/F12 (Cat#12634-010, Thermo Fisher Scientific) supplemented with 1 mM Glutamax, 50 µM β-mercaptoethanol, 50 U/ml penicillin/streptomycin, 100 ng/ml IL34 (Cat#200-34, Peprotech) and 10 ng/ml GM-CSF (Cat#PHC2013, Thermo Fisher Scientific) and was changed three times per week for a total of two weeks (14 DIV microglia). The differentiated microglia were subsequently collected 700 µl Qiazol solution (miRNeasy Micro Kit) and the RNA was extracted as described below.

Table 3. Information on iPSC lines

ID iPSC	Lab code	Age	Health status	Gender	APOE genotype	Race	Source
AG27611	CTR611	75	Healthy	Female	E3/E3	White	Coriell Institute
AG27602	CTR602	72	Healthy	Male	E3/E3	White	Coriell Institute
AG27605	AD605	72	Sporadic Alzheimer's disease	Male	E3/E3	White	Coriell Institute

2.2 RNA extraction

To isolate the RNA from iPSCs, the mirVana™ PARIS™ RNA and Native Protein Purification Kit (Cat#AM1556, Invitrogen) was utilized. Briefly, the cells were collected in 1 ml Trizol solution and subsequently incubated in 100% chloroform (Cat#J67241-AP, Thermo Fisher Scientific) for 5 min at room temperature. The samples were then centrifuged at 12.000 xg for 1 minute and the upper aqueous phase was collected into a new Eppendorf tube, where 1.25 times the volume of absolute ethanol was added. Next, the samples were passed through miRVana spin filter columns and centrifuged at 12000 xg for 40 s at room temperature. After consecutive washing steps the RNA was eluted with RNase free H_2O, and the concentration and purity were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.).

To extract the RNA from macrophage precursors and microglia the miRNeasy Micro Kit (Cat#217084, QIAGEN) was used. First, the cells were carefully thawed on ice. Next, the samples were vortexed for 30 s and left to rest for 5 min at RT. Then, the samples were incubated with 100% chloroform and were vigorously mixed for 15 s. Subsequently, the samples were left at room temperature for 2 min and then centrifuged at 12.000 xg at 4ºC for 15 min. After

centrifugation, upper aqueous phase was collected into a new Eppendorf tube, where 1.5 times the volume of absolute ethanol was added. The sample were then loaded into RNeasy MinElute Spin columns and centrifuged at 10000 xg for 15 s at room temperature. After consecutive washing steps the RNA was eluted with RNase free H_2O, and the concentration and purity were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.)

2.3 miRNA reverse transcription PCR (RT-PCR) and semi-quantitative real time PCR (qPCR)

Reverse transcription of miRNA was performed using the miRCURY LNA RT Kit (Cat#339340, QIAGEN). First, the RNA was diluted to a final concentration of 50 ng/µl. For each sample, 2 µl of RNA (100 ng) was added to 2 µl 5x Reaction buffer Sybr Green, 4.5 µl Nuclease free water, 1 µl Enzyme mix and 0.5 µl Synthetic RNA spike ins. The samples were then thoroughly mixed, spun down and incubated at 42°C for 60 min and 95°C for 5 min in the T100™ Thermal Cycler (BioRad). After the samples had cooled down to 4°C they were stored at -20°C.

miRNA gene expression levels were detected with real-time semi-quantitative PCR using the Sybr Green mastermix (Cat#1708880, QIAGEN) and miRCURY LNA primers (Cat#204129, QIAGEN). 3.5 µl of cDNA were added to a 384-well plate (Applied BioSystems) along with a mixture of 5 µl miRCURY SYBR® Green PCR Master Mix (QIAGEN), 0.05 µl ROX™ reference dye (QIAGEN) and 1 µl Forward and Reverse Primer Mix (10 pmol/µl). The plates were run in the Quantstudio 5 Real-Time PCR system. For data analysis the mean expression of either two or three small-RNA housekeeping genes (U6 snRNA, RNU5G and/ or SNORD48) was used for normalization. Fold changes were calculated using the 2-ΔΔCT method as previously described (Livak & Schmittgen, 2001). Primer sequences and relative information can be found in Table 2.

2.4 mRNA reverse transcription PCR (RT-PCR) and semi-quantitative real time PCR (qPCR)

Reverse transcription of mRNA was performed using the Superscript II reverse transcriptase (Cat#18064071, Thermo Fisher Scientific). First, the RNA was diluted to a final concentration of 50 ng/µl. For each sample, 4 µl of RNA (200 ng) were added to 1 µl Oligo(dT)12-18 (500 µg/ml) (ThermoFisher), 1 µl dNTP mix (10 mM each) (ThermoFisher) and 6 µl nuclease free water. The samples were then thoroughly mixed, spun down and incubated at 65°C for 5 min

in the T100™ Thermal Cycler (BioRad). Next, 1 µl of SuperScript II RT (LifeTechnologies) was added and then the samples were placed in the T100™ Thermal Cycler (BioRad) for 50 min at 42°C for and subsequently for 15 min at 70°C. After the samples had cooled down to 4°C they were stored at -20°C.

mRNA gene expression levels were detected with real-time semi-quantitative PCR using the PowerUp SYBR Green Master Mix (Cat#A25741, Applied BioSystems). 5 µl of cDNA were added to a 384-well plate (Applied BioSystems) along with 2 µl of Forward and Reverse Primer Mix (10pmol/µl), 3 µl Nuclease free water and 10 µl of PowerUp SYBR Green Master Mix. The plates were run in the Quantstudio 5 Real-Time PCR system. For data analysis the mean expression of two housekeeping genes (UBC and RPLP0) was used for normalization. Fold changes were calculated using the 2-ΔΔCT method as previously described (Livak & Schmittgen, 2001). Primer sequences and relative information can be found in Table 2.

Table 2. Primer sequences and relative information

Primer	Forward	Reverse
SOX2	TGGACAGTTACGCGCACAT	CGAGTAGGACATGCTGTAGGT
OCT4	GAGGAGTCCCAGGACATCAA	AGATGGTCGTTTGGCTGAAT
HEXB	GGGAGCATTACGAGGTTTAGAG	GGTGGATTCATTGATGGTGAAAG
MERTK	AGCGGGAGATCGAGGAGT	CCCCGTATTTCATGAAGGGTA
PROS	AAGAAGCCAGGGAGGTCTTTG	ACGTGCAGCAGTGAATAACC
APOC1	TGGTTCTGTCGATCGTCTTG	GAAAACCACTCCCGCATCT
CD9	TTCCTCTTGGTGATATTCGCCA	AGTTCAACGCATAGTGGATGG
LGALS3	GTGAAGCCCAATGCAAACAGA	AGCGTGGGTTAAAGTGGAAGG
UBC	GGGTCGCAGTTCTTGTTTGT	GGAGGGATGCCTTCCTTATC
RPLP0	CCTCGTGGAAGTGACATCGT	CTGTCTTCCCTGGGCATCAC
APOE	GTTCTGTGGGCTGCGTTG	AATCCCAAAAGCGACCCAGT
Primer	**Sequence**	**Cat.number (Cat#)**
hsa-miR-132-3p	UAACAGUCUACAGCCAUGGUCG	339306, QIAGEN
mmu-miR-212-3p	UAACAGUCUCCAGUCACGGCC	339306, QIAGEN
U6 snRNA (hsa, mmu)		339306, QIAGEN
RnU5G (hsa, mmu)		339306, QIAGEN
SNORD48 (hsa)		339306, QIAGEN

2.5 Immunocytochemistry (ICC)

Differentiated microglia (14 DIV) were fixated on coverslips using 4% PFA (Thermo Scientific) and stored at 4°C until further use. Firstly, the fixated cells were washed three times with 1% (v/v) TritonX-100 (Sigma-Aldrich) in PBS 1x (PBS-T) for 5 min. Subsequently, the cells were incubated with a blocking buffer solution that consisted of 1% (v/v) TritonX-100 and 10% (v/v) goat serum (Jackson ImmunoResearch) in PBS-T, for 2 hours at room temperature with constant

shaking. Next, the cells were incubated with the primary antibody solution (primary antibodies in 3% goat serum in PBS 1x and 0.3% TritonX-100, Table 3) at 4 °C in a humidified shaking chamber overnight. The following day, the cells were washed two times with PBS-T for 5 min and then incubated with the secondary antibody solution (secondary antibodies in 3% goat serum in PBS 1x and 0.3% TritonX-100, Table 3), on a shaker for 2 hours, at room temperature in the dark. After the incubation with the secondary antibody solution, the cells were washed three times with PBS-T for 5 min and then the nuclei of the cells were stained with DAPI in PBS 1x (1:5000, Sigma-Aldrich) for 10 min in the dark. Lastly, two five-minute washes were performed with PBS 1x and the coverslips were mounted onto glass microscopy slides using a small amount of Mowiol mounting medium [0.1 M Tris pH 8.5, 25% glycerol (Sigma-Aldrich), 10% MOWIOL (Merck Millipore) and 2.5% DABCO (Sigma-Aldrich)]. Once the slides had dried, they were stored at 4°C until further use. More information about the primary and secondary antibodies that were utilized is included in Table 3 below.

Table 3. Antibody information.

Primary antibodies	**Dilution**	**Cat. number (Cat#)**	**Supplier**
Goat anti-Iba1	1:100	NB 100-1028	Novus Biologicals
Rabbit anti TMEM119	1:100	AB185333	Abcam
Secondary antibodies	**Dilution**	**Cat. number (Cat#)**	**Supplier**
Donkey anti-goat 594	1:500	A11058	Invitrogen
Goat anti-rabbit 488	1:500	A11039	DyLight

2.6 Image acquisition, processing, and analysis

Immunofluorescent images [Format: 1024 x 1024; Speed: 200; Image size: 276.79*276.79 µm; Optical section: 1.194 µm; Number of Z-stack steps: 10; Line accumulation: 1 (DAPI) and 2 (TMEM119 and IBA1), Sequential Scan: Between Stacks] were acquired with the DMI6000 CS Confocal microscope (Leica) at 40x magnification, using the LAS X Office software. For DAPI (Blue, UV 405, Laser power: 8.05%) the spectral window was set to 415-480 nm, for TMEM119 (Green, WLL 488, Laser power: 23.4%) to 498-560 nm and for Iba1 (Red, WLL 594, Laser power: 51%) to 604-670 nm. For the detection the HyD spectral detector was utilized with photon counting.

Sholl analysis of microscopy images was executed with Fiji (ImageJ) using the Sholl Analysis plugin with a 1 µm step size from the cell soma. Quantification of microglial cells was executed in confocal images with Fiji (ImageJ) using the StarDist plugin.

2.7 Quantification of gene expression and statistical analysis

Statistical calculations were performed with Prism (v10.0.0 GraphPad). The data are presented as the mean expression ± SD. The significance was set at (adjusted) p-value threshold of 0.05 and was calculated using one-way ANOVA after post hoc Bonferroni correction. Outlier values were removed according to the ROUT test with Q set to 1%. Correlation analyses were performed using Pearson's correlation (two-tailed, 95% confidence interval) and simple linear regression of correlation. To improve the appearance of the Sholl analysis graph the data were smoothed using the Smooth tool in Prism (v10.0.0 GraphPad).

CHAPTER 3. Results

3.1 Downregulation of pluripotency markers and upregulation of microglial markers upon microglial differentiation from human iPSCs

To characterize the iPSC lines of interest at baseline, we first conducted qPCR analysis to detect and quantify the expression levels of specific pluripotency (*OCT4, SOX2*) (Takahashi & Yamanaka, 2006) and microglial markers (*HEXB, MERTK, PROS1*) (Butovsky et al., 2014) (Figure 3). This crucial process was employed to confirm the cellular identity of the cells under investigation, ensuring that their expression profile resembled that of microglia-like cells in our experimental setting, thereby verifying the efficiency of our reprogramming protocol.

By comparing the different cell types within each line, we found a statistically significant downregulation of pluripotency markers and an upregulation of microglial markers in the differentiated microglia-like cells across all lines (Figure 3). Specifically, CTR611 microglial differentiation resulted in a ~18-fold change increase of *HEXB*, ~17-fold change increase of *MERTK* and ~27-fold change increase of *PROS1* compared to iPSCs (Figure 3A). CTR602 microglial differentiation resulted in a ~10-fold change increase of *HEXB*, ~33-fold change increase of *MERTK* and ~10-fold change increase of *PROS1* compared to iPSCs (Figure 3B). Lastly, AD605 microglial differentiation resulted in a ~16-fold change increase of *HEXB*, ~17-fold change increase of *MERTK* and ~20-fold change increase of *PROS1* compared to iPSCs (Figure 3C).

These observations indicate the successful differentiation of the iPSCs into microglia-like cells, henceforth referred to as microglia. Upon closer examination of the absolute fold change of the various markers, a noticeable variability was observed across the different cell lines.

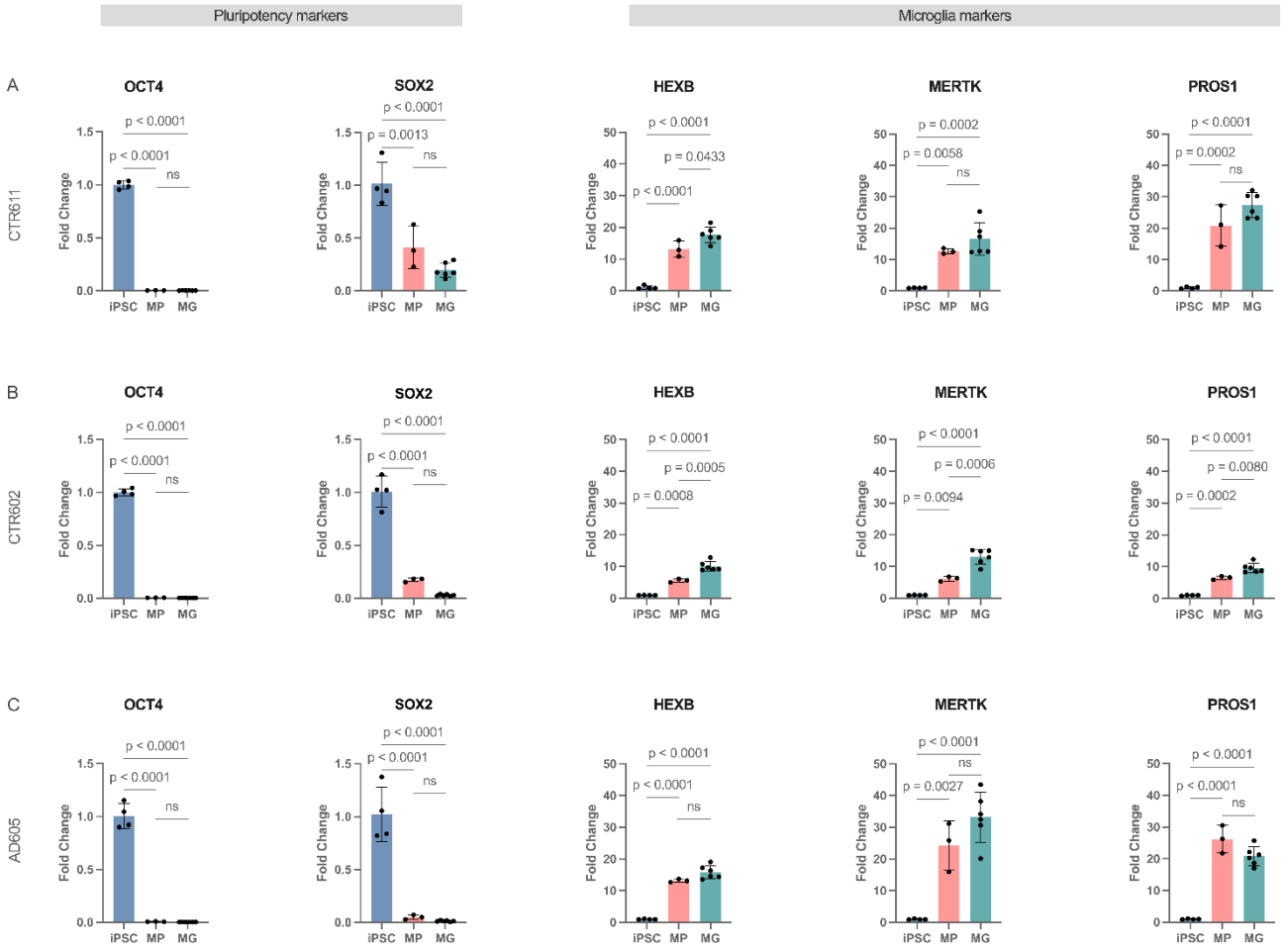


Figure 3. **Downregulation of pluripotency markers and upregulation of microglial markers upon microglial differentiation from human iPSCs**. **A, B, C**. Semi-quantitative real-time PCR of pluripotency and microglial marker levels upon differentiation from iPSCs to microglia. N = 3-6 technical replicates. CTR, control; AD, Alzheimer's disease; iPSCs, induced pluripotent stem cells; MPs, macrophage precursors; MG, microglia. Bar plots represent the mean expression of each gene ± SD for each cell type. Statistical significance was calculated using one-way ANOVA after post hoc Bonferroni correction. ns, not statistically significant.

3.2 Expression profiles of miR-132 and miR-212 upon microglial differentiation from human iPSCs

As mentioned, miR-132 has been shown to be consistently and robustly downregulated in Alzheimer's disease (Lau et al., 2013) and is considered a promising therapeutic target for battling AD (El Fatimy et al., 2018; Salta & De Strooper, 2017). In a recent study, we reported the upregulation of miR-132 expression levels along microglial differentiation in a commercially available control iPSC line (Sigma Ctrl iPSCs P5 24/7/21, Cat# IPSC0028) (Walgrave et al., 2023). With the aim of expanding our investigation, we focused on implementing three additional iPSC lines (CTR611, CTR602 and AD605, see Table 1). To this end, we monitored their miR-132 expression levels across microglial differentiation. For a more comprehensive analysis we also investigated the expression levels of miR-212, a cognate miRNA to miR-132 (Wanet et al., 2012).

When comparing the different cell types within each line (Figure 2A), we observe that both miR-132 and miR-212 were significantly upregulated in differentiated microglia in CTR611 (~33-fold change increase of miR-132 and ~53-fold change increase of miR-212 comparing to iPSCs) and AD605 (~32-fold change increase of miR-132 and ~60-fold change increase of miR-212 comparing to iPSCs) lines (Figure 4A, C). Interestingly, despite an apparent trend, the expression levels of miR-132 was not found to be significantly upregulated in differentiated microglia in the CTR602 line (Figure 4B). Similarly to the previous results, the absolute fold change of miR-132 and miR-212 expression levels demonstrates a noticeable variability between the different lines.

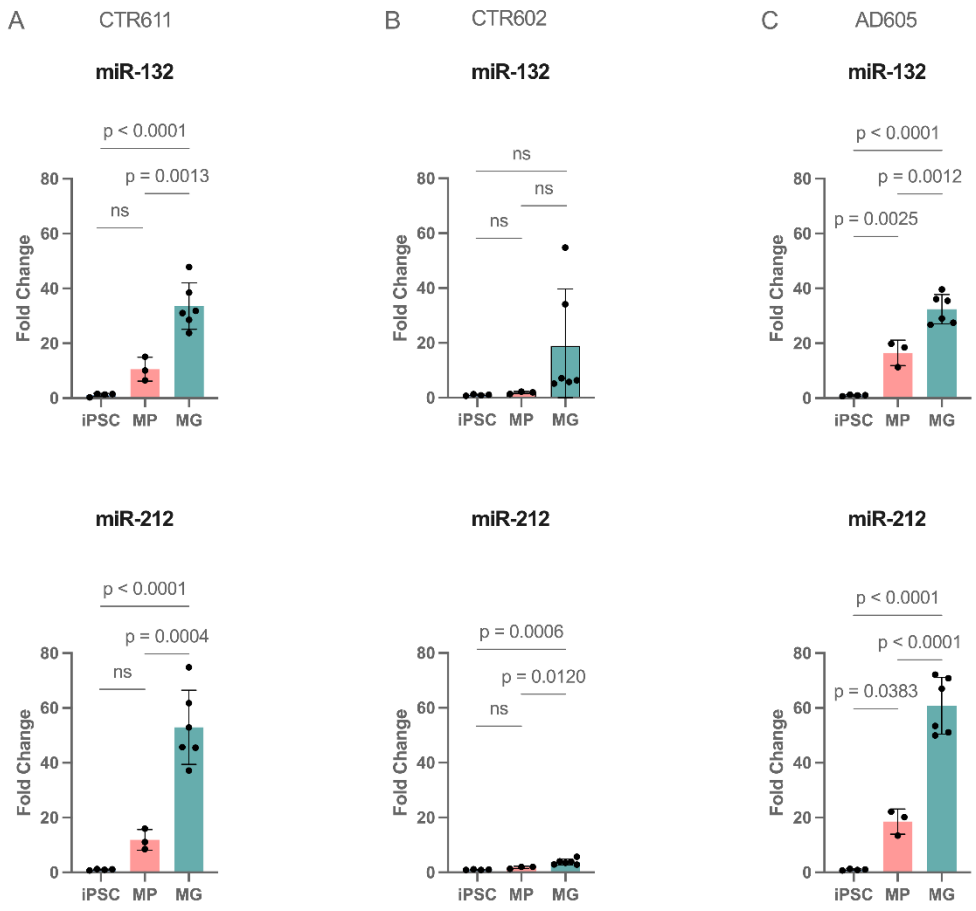


Figure 4. **Expression levels of miR-132 and miR-212 upon microglial differentiation from human iPSCs.** A, B, C. Semi-quantitative real-time PCR of miR-132 and miR-212 levels upon differentiation from human iPSCs to microglia. N = 3-6 technical replicates. miR-212 was used as a control. CTR, control; AD, Alzheimer's disease; iPSCs, induced pluripotent stem cells; MG, microglia; miR-132, microRNA-132; miR-212, microRNA-212. Bar plots represent the mean expression of each gene ± SD for each cell type. Statistical significance was calculated using one-way ANOVA after post hoc Bonferroni correction. ns, not statistically significant.

3.3 Expression profiles of DAM and AD risk genes across distinct cell lines

A plethora of studies have identified distinct subpopulations of microglia, which can be distinguished by their unique transcriptomic signature (Chen & Colonna, 2021). One microglial subpopulation that expresses many AD risk genes, such as *APOE* and *TREM2*, is the disease-associated microglia, generally referred to as DAM (Hansen et al., 2018; Keren-Shaul et al., 2017). Thus, to profile microglial states, we next monitored the expression levels of specific DAM genes (*CD9*, *LGALS3* and *APOC1*, Figure 5A) between the different lines as well as between different conditions (Figure 2B, C respectively). Since *APOE* constitutes the largest risk factor for sporadic AD, its expression levels were also investigated (Figure 5B).

Intriguingly, the DAM expression levels of CTR602 resembled that of AD605, with significantly higher expression compared to CTR611 (Figure 5A). Further, *APOE* showed a significant upregulation in AD605, after the removal of one computed outlier, compared to CTR611, but not CTR602 (Figure 5B). *APOE* expression levels showed a similar expression pattern between the two controls. As expected, all microglia expressed the *HEXB* and *MERTK* microglial markers across the different cell lines (Figure 5C).

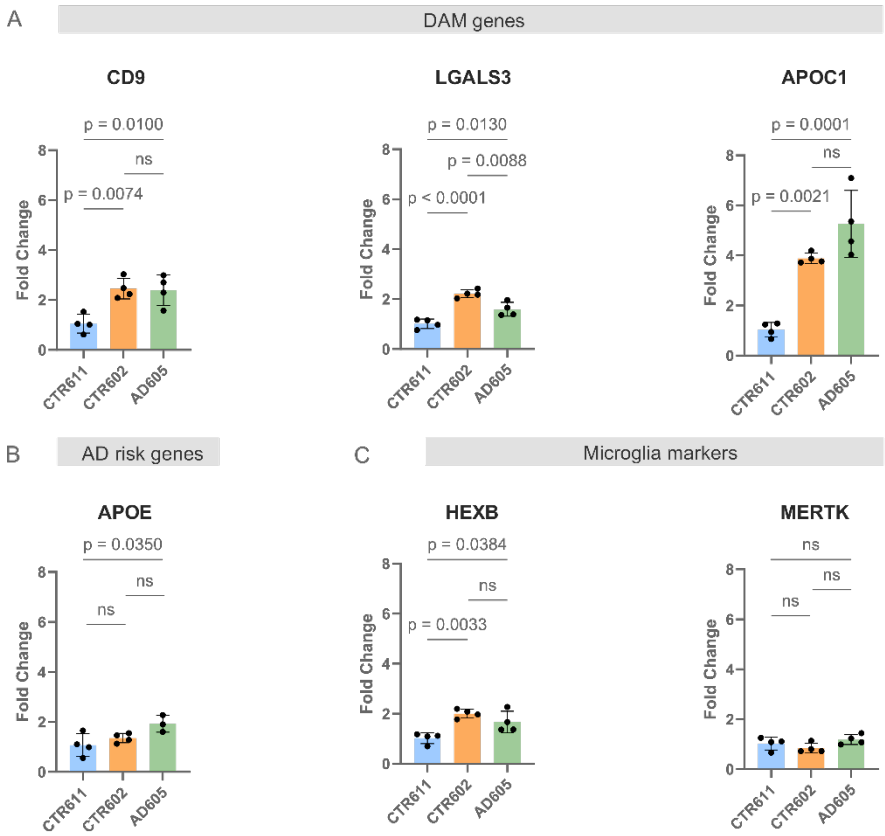


Figure 5. **Expression levels of DAM and AD risk genes across distinct cell lines**.
A, B, C. Semi-quantitative real-time PCR of DAM gene (A), AD risk gene (B) or microglial marker (C) levels in differentiated microglia across different cell lines. N = 4 technical replicates. CTR, control; AD, Alzheimer's disease; DAM, disease-associated microglia. Bar plots represent the mean expression of each gene ± SD for each line. Statistical significance was calculated using one-way ANOVA after post hoc Bonferroni correction. Outlier values were removed according to the ROUT test. ns, not statistically significant

3.4 Expression profiles of miR-132 and miR-212 across distinct cell lines

Subsequently, we monitored the expression levels of miR-132 and miR-212 between the different cell lines and conditions (Figure 2B, C respectively). Notably, we observed a statistically significant decrease in the expression levels of both miR-132 and miR-212 in CTR602 compared to CTR611 and AD605 (Figure 6). Surprisingly, we did not observe the expected downregulation of miR-132 expression levels in the AD line (Figure 6). Instead, we observed similar expression levels of miR-132 and miR-212 between CTR611 and AD605.

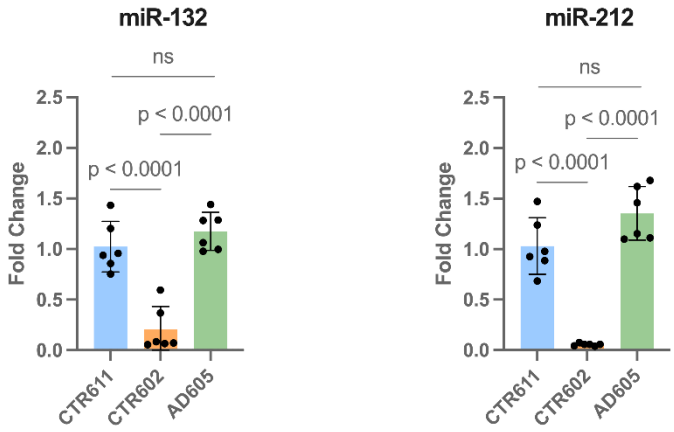


Figure 6. **Expression levels of miR-132 and miR-212 across distinct cell lines.** A. Semi-quantitative real-time PCR of miR-132 and miR-212 levels in differentiated microglia across different cell lines. N = 6 technical replicates. miR-212 was used as a control. CTR, control; AD, Alzheimer's disease; miR-132, microRNA-132; miR-212, microRNA-212. Bar plots represent the mean expression of each gene ± SD for each line. Statistical significance was calculated using one-way ANOVA after post hoc Bonferroni correction. ns, not statistically significant

3.5 Housekeeper selection impacts the computed statistical significance and fold change in data analysis

Identifying and validating proper housekeeping genes, the combination of which can be used for proper internal normalization of gene expression data in any system is critical. For data analysis and normalization the mean expression of two small-RNA housekeeping genes (*U6* snRNA and *RNU5G*) was ultimately employed. Interestingly, we observed that different housekeeper combinations (*U6* snRNA, *RnU5G*, *SNORD48*) can influence the normalization process, affecting the relative expression levels of miR-132 and miR-212 (Figure 7A-D). Consequently, the calculated p-values, which indicate the statistical significance of the observed differences, can also be influenced by the choice of housekeepers.

Specifically, when *U6* or *RnU5G* housekeepers were combined with *SNORD48*, the observed decrease of miR-132 expression levels in CTR602 was no longer significant (Figure 7B, C). This striking observation highlights, how crucial it is to carefully select and validate the appropriate combination of housekeepers to ensure accurate and reliable data analysis.

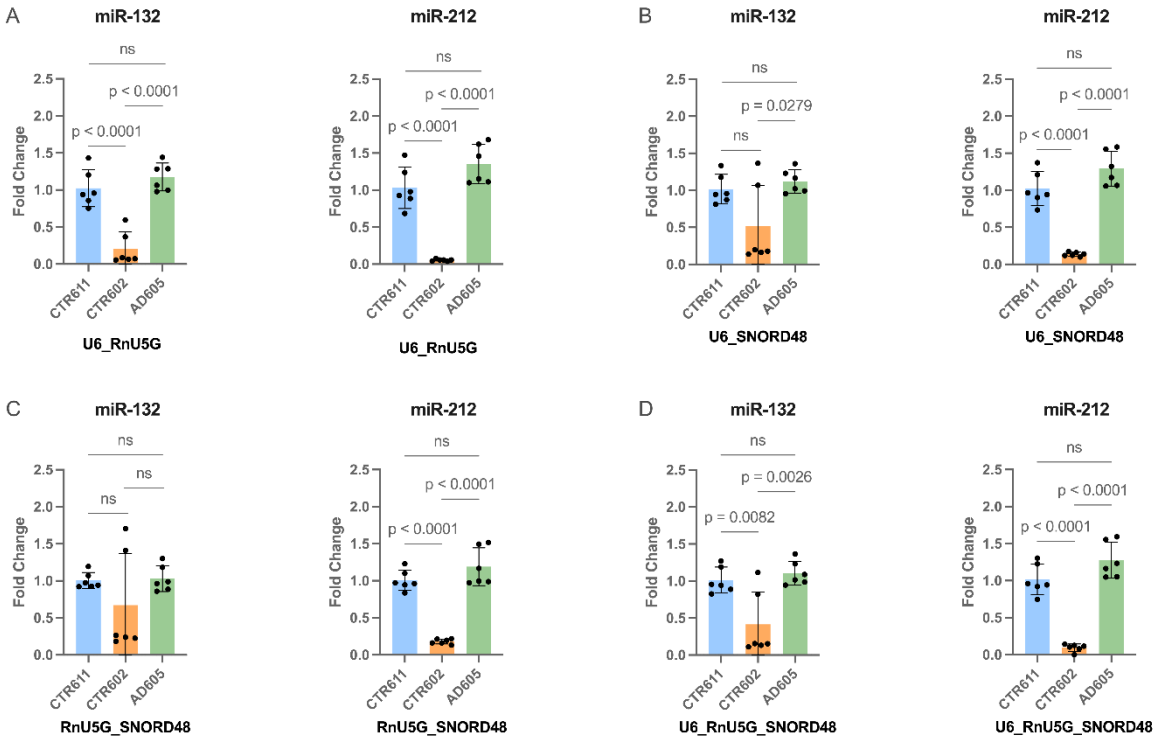


Figure 7. **Housekeeper selection impacts the computed statistical significance and fold change in data analysis**.
A-D. Different combinations of housekeepers (*U6*, *RnU5G* and *SNORD48*) significantly impact the findings derived from the analysis. The expression levels of miR-132 and miR-212, as well as the significance (p-value) of the observed differences are differentially affected by the combination of housekeepers used for the analysis. Semi-quantitative real-time PCR of miR-132 and miR-212 levels in differentiated microglia across different cell lines. N = 6 technical replicates. CTR, control; AD, Alzheimer's disease; miR-132, microRNA-132; miR-212, microRNA-212. Bar plots represent the mean expression of each gene ± SD for each line. Statistical significance was calculated using one-way ANOVA after post hoc Bonferroni correction. ns, not statistically significant.

3.6 Distinct microglial morphology in sAD patients

It is widely recognized that homeostatic microglia adopt a more ramified morphology, with longer processes continuously surveying their environment. However, upon detection of a stimulus or under pathophysiological conditions, such as AD, microglia take on a more "activated" amoeboid shape with shorter processes (Lin et al., 2018; Parakalan et al., 2012). To investigate the morphological features of microglia, we performed immunocytochemistry stainings in PFA-fixed coverslips, using microglia-specific antibodies (IBA1 and TMEM119) (Figure 8A). This approach allowed us to examine the structural characteristics of microglia and gain insights into their morphology and activation status.

Sholl analysis revealed distinct morphological characteristics in the sAD iPSC line compared to controls. Collectively, microglia derived from the control iPSC lines demonstrated a more complex morphology compared to the sAD iPSC line, as indicated by the area under the curve (AUC) of the Sholl analysis (Figure 8B, C). The peak of the AUC (maximum number of intersections) was significantly higher in the first control compared to the sAD iPSC line (Figure 8B, D). Interestingly, we did not observe any statistically significant differences in the median number of branches between the lines, further supporting the notion of an overall more activated morphological phenotype in cultured cells (Gumbs et al., 2022; Timmerman et al., 2018) (Figure 8E). iPSC-derived microglia from both control lines demonstrated longer processes (wingspan) compared to the sAD iPSC line, although for the second control this did not reach statistical significance (Figure 8F).

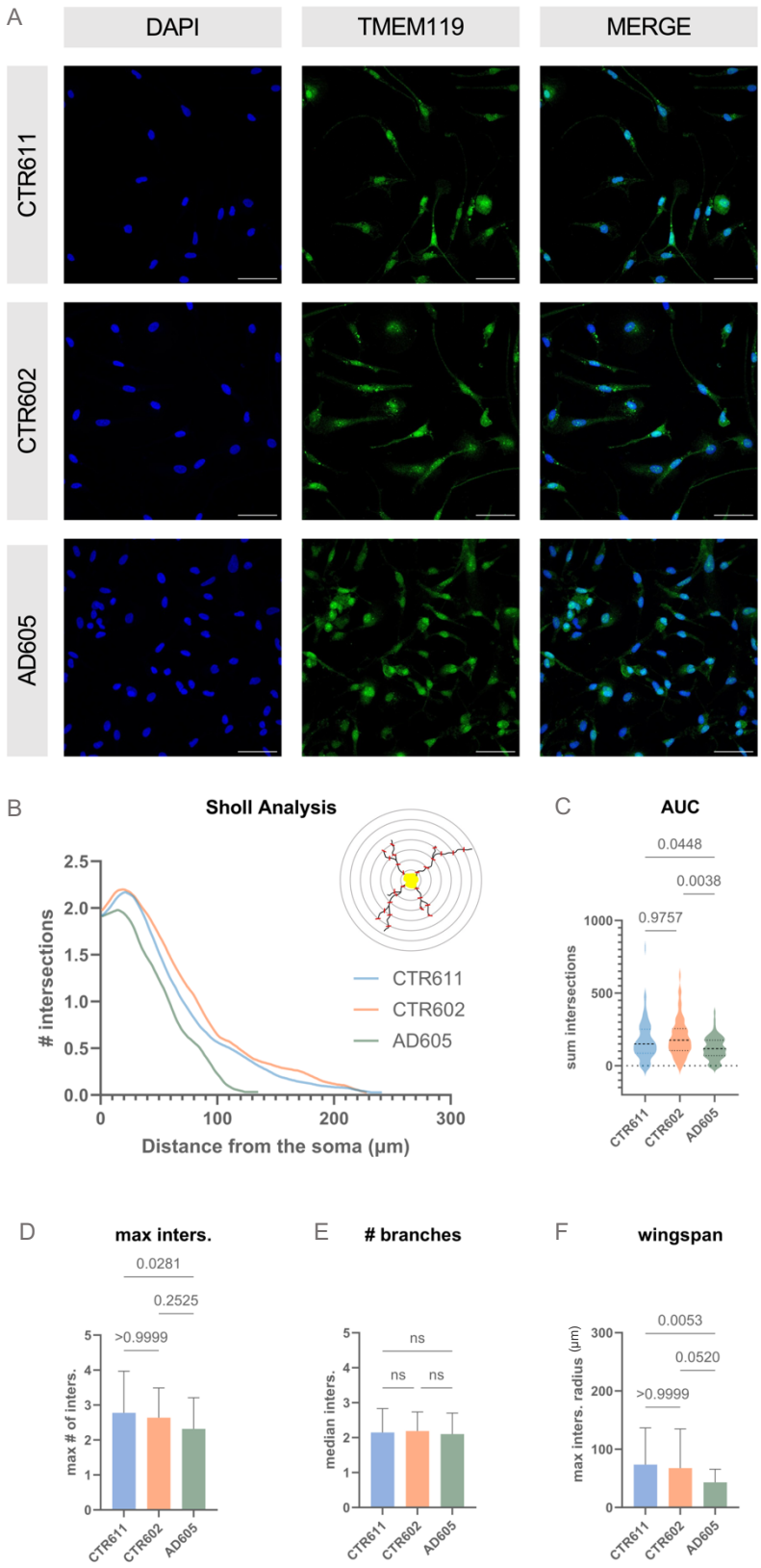


Figure 8. **Distinct microglial morphology in sAD patients.**

A. Immunolabelling of TMEM199 (green) and DAPI (blue) in iPSC-derived microglia from CTR611, CTR602 and AD605 lines. Merge indicates the overlap of the different channels. Scale bars = 50 µm. **B**. Sholl analysis of the average number of microglial branch intersections with a 1 µm step from the cell soma. **C**. Sholl-derived AUC per microglia. **D**. Quantification of the maximum number of intersections in each iPSC line. **E**. Quantification of the median number of intersections in each iPSC line. **F**. Quantification of the maximum branch extent in µm from the cell soma in each iPSC line. n = 72 microglia in CTR611, n = 58 microglia in CTR602, n = 60 microglia in AD605. Bar plots represent the mean expression of each gene ± SD for each line. Statistical significance was calculated using one-way ANOVA after post hoc Bonferroni correction. ns, not statistically significant. CTR, control; AD, Alzheimer's disease AUC, area under the curve; inters, intersections; Scale bars = 50 µm

CHAPTER 4. Discussion

Microglia are the innate immune cells of the central nervous system. Over the past years, several GWAS studies have identified AD risk genes that are highly expressed in microglia, shedding light on the contribution of dysfunctional microglia in the pathogenesis of AD (Lambert et al., 2013, 2023; Tábuas-Pereira et al., 2020). miR-132 has been shown to be consistently and robustly downregulated in Alzheimer's disease patients (Lau et al., 2013) and restoration of miR-132 expression levels in AD animal models has been demonstrated to rescue memory deficits (Walgrave, Balusu, et al., 2021), thus rendering it a promising therapeutic target for battling AD (Salta & De Strooper, 2017, PMID: 34033742). Notably, even though the vast majority of studies have reported miR-132 regulatory roles in neuronal systems, we recently demonstrated a novel miR-132 mediated effect over microglial activation (Walgrave et al., 2023). Importantly, before considering any therapeutic approaches that involve the supplementation of miR-132, it is imperative to thoroughly define the broad functional effects of miR-132 in the human context. Here, by monitoring gene expression profiles using quantitative PCR and immunofluorescent labelling we systematically characterized iPSC-derived microglia from two healthy individuals and one sAD patient. We demonstrated that iPSC-derived microglia highly express microglial markers, thereby validating their cellular identity in our experimental setting. In addition, we showed an upregulation of DAM and AD risk genes in the sAD iPSC line, and we observed distinct morphological characteristics in the sAD iPSC line compared to controls, which may suggest a more activated phenotype. Interestingly, despite these observations no downregulation of miR-132 expression levels in the sAD iPSC line was observed.

The development of microglial differentiation protocols from human iPSCs has been challenging. It is only recently that researchers have been able to mimic the in vivo development of microglia in vitro and since then a plethora of protocols have been established (Abud et al., 2017; Brownjohn et al., 2018; Douvaras et al., 2017; Dräger et al., 2022; Haenseler et al., 2017). To validate the cellular identity of the differentiated microglia in this study, microglial markers that had been previously proposed as microglia-specific were employed (Butovsky et al., 2014). The results demonstrated a clear upregulation of differentiation markers and downregulation of pluripotency markers across all lines, confirming the microglial identity of the cells. However, microglia are often referred to as the resident macrophages of the CNS, thereby reflecting the functional similarities of the two cell types. Thus, it is important to note that these markers can also be expressed by macrophages and therefore caution should be applied when validating the

cellular identity of iPSC-derived microglia solely based on their marker expression (Jurga et al., 2020). For a more comprehensive analysis, follow-up functional assays should also be considered.

DAMs are a microglial subtype that express many AD risk genes, including *APOE*, the major risk factor for sporadic AD (Hansen et al., 2018; Keren-Shaul et al., 2017). Under physiological conditions, *APOE* is mainly synthesized and secreted by astrocytes. In pathological conditions however, DAMs exhibit a significant increase in *APOE* expression (Keren-Shaul et al., 2017; Krasemann et al., 2017). Consistent with these findings, our study revealed elevated *APOE* and DAM-associated gene expression levels in sAD iPSC-derived microglia compared to the microglia of the second iPSC control line (CTR602). Interestingly, despite both control lines showing similar *APOE* expression levels (all iPSC lines are homozygous for the APOE3 allele), the expression levels of DAM associated genes were similarly increased between the microglia derived from second control line and the microglia derived from the sAD iPSC line. The increased DAM gene signatures in the sAD iPSC line are in agreement with the more pronounced inflammatory phenotype that has been reported in sAD microglia (M. Xu et al., 2019), while the differences between the control lines could either be attributed to interindividual variability or differences in the experimental conditions (i.e CTR602: poor sample quality). Considering the possibility that higher sample quality might have contributed to a more robust outcome, we cannot exclude this as a confounding factor impacting the observed results. Nevertheless, our data show a similar upregulation of DAM gene signatures in the second control and sAD iPSC line, suggestive of a more activated transcriptional profile.

Microglial morphology is highly complex, with microglia constantly reorganizing their structure in response to environmental stimuli (Nimmerjahn, Kirchhoff, & Helmchen, 2005). Homeostatic microglia adopt a more ramified morphology, with longer processes continuously surveying their environment. Upon detection of a stimulus, microglia take on a more "activated" amoeboid shape with shorter and fewer processes, that is characterized by the secretion of a battery of proinflammatory cytokines (Parakalan et al., 2012). In vivo microglia display notable differences in their morphological features compared to microglia cultured in vitro, with the latter appearing more "activated", while secreting a greater number of proinflammatory cytokines at baseline (Gumbs et al., 2022; Timmerman et al., 2018). In the context of sporadic AD, *APOE4* iPSC-derived microglia exhibited fewer processes compared to their isogenic *APOE3* control iPSC-derived microglia (Lin et al., 2018). In line with these observations, our data show a clear reduction in the sum number of intersections in the sAD

iPSC-derived microglia compared to the control iPSC lines, suggestive of a more activated phenotype, which is further supported by the observed elevated expression of DAM gene signatures. Interestingly, the second control did not demonstrate the same morphological characteristics as the sAD iPSC line, despite the elevated DAM gene expression signatures that were observed. Thus, the transcriptional data suggest that the second control iPSC line appears to be more activated than the first control line, despite both lines showing similar morphological characteristics. Considering that the *APOE* expression levels were not increased in the second control iPSC line, this could perhaps indicate that high *APOE* expression is necessary for a more activated morphological phenotype, similar to the sAD iPSC line. This is further supported by a previous study, which demonstrated that suppression of the APOE pathway can restore the homeostatic microglial phenotype in an AD mouse model (Krasemann et al., 2017). Nonetheless, further research and higher sample sizes are required to fully comprehend the underlying mechanisms driving microglial morphological changes and their relationship with *APOE* expression levels in the context of neurodegenerative diseases.

Although miR-132 has been shown to be consistently and robustly downregulated in Alzheimer's disease (Lau et al., 2013), our results demonstrate similar expression levels of miR-132 in sAD iPSC-derived microglia compared to the microglia derived from the first control line (CTR611). It is worth mentioning that the differentiation of iPSC-derived microglia is a complex and timely sensitive procedure. Studies in iPSC-derived neurons have demonstrated that the expression of TAU isoforms follows a temporal developmental pattern, with only the fetal TAU isoform detected during early differentiation stages, while all six TAU isoforms are expressed as neurons mature over the course of several months (Iovino et al., 2015; Sposito et al., 2015). What is more, it has been suggested that sAD iPSC-derived microglia exhibit early-stage AD based on their increased phagocytic ability that is present in the initial stages of AD (M. Xu et al., 2019). Similarly, miR-132 expression undergoes distinct changes throughout the course of AD progression. During the early Braak stages in AD there is an upregulation in the expression levels of miR-132 and as the disease progresses a robust downregulation is observed (Lau et al., 2013). Given these observations, one possible explanation for the similar miR-132 expression levels between the sAD line and the first control line could be the young age of the factories from which the macrophage precursors were harvested. These findings highlight the importance of considering the temporal aspects of iPSC-derived microglial differentiation, as the time of harvesting and the maturity of the factories can have a profound impact on miRNA expression regulation.

Another explanation for the observed results could be interindividual variability. Each individual's genetic background and epigenetic modifications could potentially introduce differences in miRNA expression profiles, including miR-132. In addition, it is worth mentioning that the observed robust and consistent decrease of miR-132 expression in the human AD brain was primarily driven from neurons rather than microglia (Lau et al., 2013) and could explain why the downregulation of miR-132 expression was not observed in our experimental setting. Considering these factors is crucial for accurate interpretation of miRNA expression data and understanding the complex regulatory landscape of miR-132 in the context of AD and microglial biology.

Recently, it has been demonstrated that miR-132 overexpression shifts microglia from a DAM to a homeostatic state (Walgrave et al., 2023). Intriguingly, here we have shown a decrease in miR-132 expression levels in the second control iPSC line, which was accompanied by an increase in the DAM gene expression signatures. These differences between the two control lines could be attributed to interindividual variability, however what makes our observations particularly interesting is the fact that even though the second control line displays a more activated expression profile, it morphologically resembles more the first control line rather than the sAD line. These findings suggest that additional intrinsic or experimental factors may influence the morphological characteristics of microglia and further research is needed to elucidate the underlying mechanisms.

In this study, we have clearly demonstrated the importance of several critical factors that can significantly impact the consistency and robustness of our results. Specifically, we have highlighted the significance of housekeeper selection, experimental design, sample quality and experiment execution. Firstly, the meticulous selection of appropriate housekeeping genes is crucial for accurate normalization of gene expression data. We have showcased that different combination of housekeepers can influence the computed statistical significance and fold change values of the observed results, although the degree to which the quality of the samples and the experiment execution might have affected this influence is yet to be addressed. Secondly, it has become apparent that maintaining a consistent experimental design and conditions throughout the study minimizes potential confounding factors that could arise from procedural discrepancies. Lastly, the inconsistent results in the expression levels of miR-132 between the baseline and treatment experiments (batch #1 and batch #2 respectively, see Supplementary figures and material) have effectively demonstrated the significance of maintaining a consistent experimental protocol that allows for constructive comparisons to be

made. Adequate sample size is another key variable in iPSC-based experimental design, since inter-individual variability may also impact data and data interpretation.

CHAPTER 5. Limitations

This study presents several limitations. Firstly, the aim of this study was to implement seven iPSC lines in total. However, due to challenges with the culturing conditions, only three iPSC lines were eventually employed, leading to limited sample size and statistical power that could have influenced the robustness of certain results. This could explain, for instance, the impact of housekeeper selection on the significance and fold change in data analysis.

Secondly, it is widely appreciated that along with the advanced age and the APOE4 genotype, female sex is regarded as one of the most significant risk factors associated with the development of sporadic AD (Farrer et al., 1997). While our study employed iPSC lines from both male and female donors, due to the limited sample size only one male sAD iPSC line was included in the analysis. Thus, we cannot draw any definitive conclusions regarding the potential influence of donor sex as a confounding factor in the observed outcomes.

Thirdly, the utilization of iPSC technology presents several limitations that should be taken into account during the interpretation of the results. These limitations primarily stem from inherent sources of variability associated with iPSC models. One significant source of variability is somatic mosaicism that can introduce variability within iPSCs derived from the same donor (Riemens et al., 2020). In this context, the origin of the tissue from which the iPSCs are generated has been demonstrated to be of great importance in determining whether the in vitro model accurately mimics the in vivo pathology (Bushman et al., 2015). Another source of variability is interindividual variability, which refers to differences observed amongst iPSC lines derived from different donors. These variations can arise from differences in genetic backgrounds or environmental influences resulting in heterogeneity within iPSC populations that can impact the interpretation of the results. Furthermore, fundamental questions regarding the preservation of epigenetic and aging traits within iPSCs persist (Riemens et al., 2020). Understanding whether iPSCs accurately retain these traits throughout reprogramming and culturing processes is essential when studying age-related diseases, such as AD.

Lastly, one major remaining issue is the translatability of in vitro findings to the in vivo context. There is no doubt that findings obtained from in vitro experiments often fail to accurately reflect the complex and dynamic nature of biological processes that occur in vivo. However, in vitro

models are the best approach to follow for reductionist studies that aim to address specific disease-related questions. In vitro models offer experimental conditions that allow researchers to study promising therapeutic targets, such as miR-132, in a human-relevant controlled environment that would be otherwise challenging to achieve in vivo.

CHAPTER 6. Conclusions

The primary objective of this study was to comprehensively characterize iPSC-derived microglia from both healthy individuals and sporadic Alzheimer's disease patients at baseline. Our findings demonstrate the successful generation of iPSC-derived microglia using a well-established differentiation protocol (Haenseler et al., 2017), while highlighting the influence of donor interindividual variability, housekeeper selection and sample quality on experimental outcomes. This study will provide fundamental knowledge for future reference, to better evaluate prospective results across different treatment conditions, that aim to shed light on the broad functional effects of miR-132 in different cell types in the human context. This will ultimately bring the scientific community one step closer to targeting the expression levels of miR-132 as an effective therapeutic approach for Alzheimer's disease.

CHAPTER 7. Future Directions

The aim of this study has been to systematically characterize iPSC-derived microglia from both healthy individuals and sporadic AD patients at baseline. However, in order to increase the power of the current study, we plan to implement a total of seven iPSC lines from both male and female donors, thereby enhancing the robustness and consistency of our results. By comparing these findings with the results obtained from similarly designed miR-132 treatment experiments, we will gain valuable insights into the specific effects of miR-132 modulation on iPSC-derived microglia.

It is widely recognised that miRNA biology is highly complex and that their ability to modulate a wide range of molecular targets is, at the same time, their biggest advantage and disadvantage (Walgrave, Zhou, et al., 2021). Therefore, before considering any therapeutic approaches involving miR-132 supplementation in the human context, it is imperative to thoroughly define the broad functional effects of miR-132, as it has been previously demonstrated that the modulation of certain miRNAs expression can have detrimental consequences (Aloi et al., 2023). Thus, we intend to perform analogous experiments in iPSC-derived neurons to gain a more comprehensive understanding of the effects of miR-132 across different cell types.

To address the limitations associated with in vitro systems, further studies ought to be carried out in co-culturing 2D and 3D systems that allow for the interaction and communication of different cell types, thereby mimicking a more physiological environment. For the same reason, conducting more studies within a more complex in vivo environment, such as studies that employ xenotransplantation approaches, are necessary to enhance the relevance and translatability of these findings (Hasselmann & Blurton-Jones, 2020; Xu et al., 2020). This will help identify any undesirable toxicity or off target effects that the exogenous supplementation of miR-132 might have, leading to the development of more effective therapeutic strategies that target the underlying causes of AD and not just the symptoms.

CHAPTER 8. Supplementary figures and material

8.1 Inconsistent results in the expression levels of miR-132 across different experiments

As it has been previously mentioned, a housekeeper-dependent statistically significant decrease was observed in the expression levels of miR-132 in CTR602 compared to CTR611 and AD605 (Figure S1A). Strikingly, no statistically significant downregulation of miR-132 expression levels was detected in the AD line. Interestingly, this observation contradicted previous findings, were miR-132 expression levels exhibited a statistically significant downregulation in the AD line (Figure S1B). Thus, we next set out to explore the source of this discrepancy.

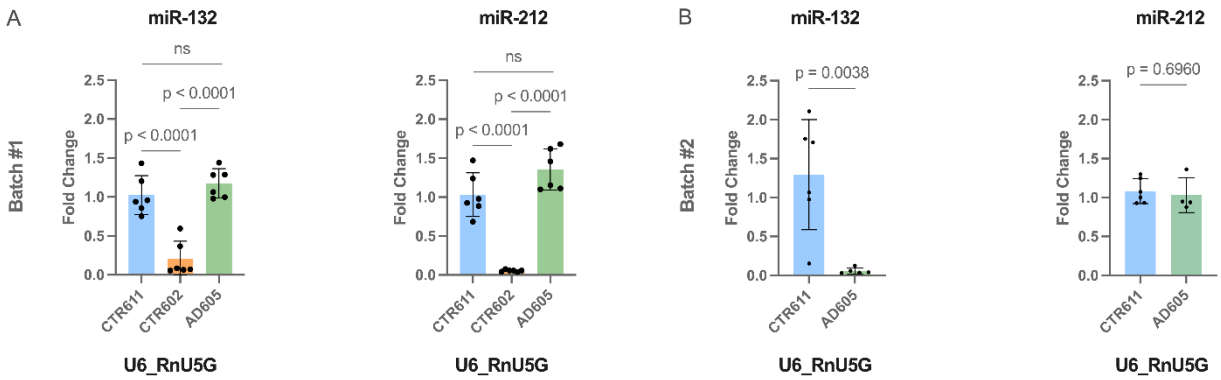


Figure S1. **Inconsistent results in the expression levels of miR-132 between different experiments.**
A. Expression levels of miR-132 and miR-212 across distinct cell lines (batch #1, no treatment). CTR602 shows decreased levels of miR-132 and miR-212 compared to CTR611 and AD605. No statistically significant differences are observed between CTR611 and AD605. Semi-quantitative real-time PCR of miR-132 and miR-212 levels in differentiated microglia across different cell lines. N = 6 technical replicates. **B**. miR-132 expression levels are significantly downregulated in AD605 compared to CTR611 (batch #2, cell lines treated with control oligonucleotides). Semi-quantitative real-time PCR of miR-132 and miR-212 levels in differentiated microglia in one control and one AD cell line. N = 5-6 technical replicates. CTR, control; AD, Alzheimer's disease; miR-132, microRNA-132; miR-212, microRNA-212. Bar plots represent the mean expression of each gene ± SD for each line. Statistical significance was calculated using one-way ANOVA after post hoc Bonferroni correction. U6 and RnU5G were used for normalization. ns, not statistically significant.

8.2 Differences in the experimental design may account for the inconsistent results observed between experiments

When taking a deeper look into the experimental design of the two experiments (batch #1 and batch #2), we observe a number of differences (Figure S2A, B). These differences could have influenced the results obtained from the two experiments. By understanding these differences, we can gain valuable insights into the factors that may have contributed to the variations in the experimental results.

Firstly, one notable difference between the experiments is that batch #2 was part of a treatment protocol. Thus, batch #2 microglia were treated with control oligonucleotides and were cultured for 20 days in vitro (DIV). In contrast, batch #1 microglia were not subjected to any kind of treatment and were cultured for 14 DIV. The differences in culturing conditions and duration could have potentially contributed to the inconsistent results for the expression levels of miR-132 and miR-212 between the two experiments.

What is more, another difference concerns the dissimilarity in cell quantity between the two batches. This is evident from the decreased number of cell clusters in batch #1 (Figure S2C, D) and could be attributed to either the shorter culturing duration or the younger age of the factories. It is worth mentioning that in this context, a cluster is defined as a significant accumulation of cells in extremely close proximity, that renders the accurate counting of the individual cells challenging. As demonstrated in Figure S2C and D, higher number of clusters correspond with higher number of cells.

Supporting this observation, the RNA concentration measurements presented in Table S1 and Table S2 are in agreement with the observed differences regarding the number of cells. Batch #1 samples exhibited low RNA concentrations (Table S1), with low number of cells, whereas batch #2 samples displayed high RNA concentrations (Table S2), with high number of cells. These factors may explain the observed differences between the two experiments. However, it is important to acknowledge that the presence of numerous differences between the two experiments at various levels, inevitably impact our ability to draw definitive conclusions. Thus, further analysis is required to understand the impact of the treatment and longer culturing duration on the expression patterns of miR-132 and miR-212.

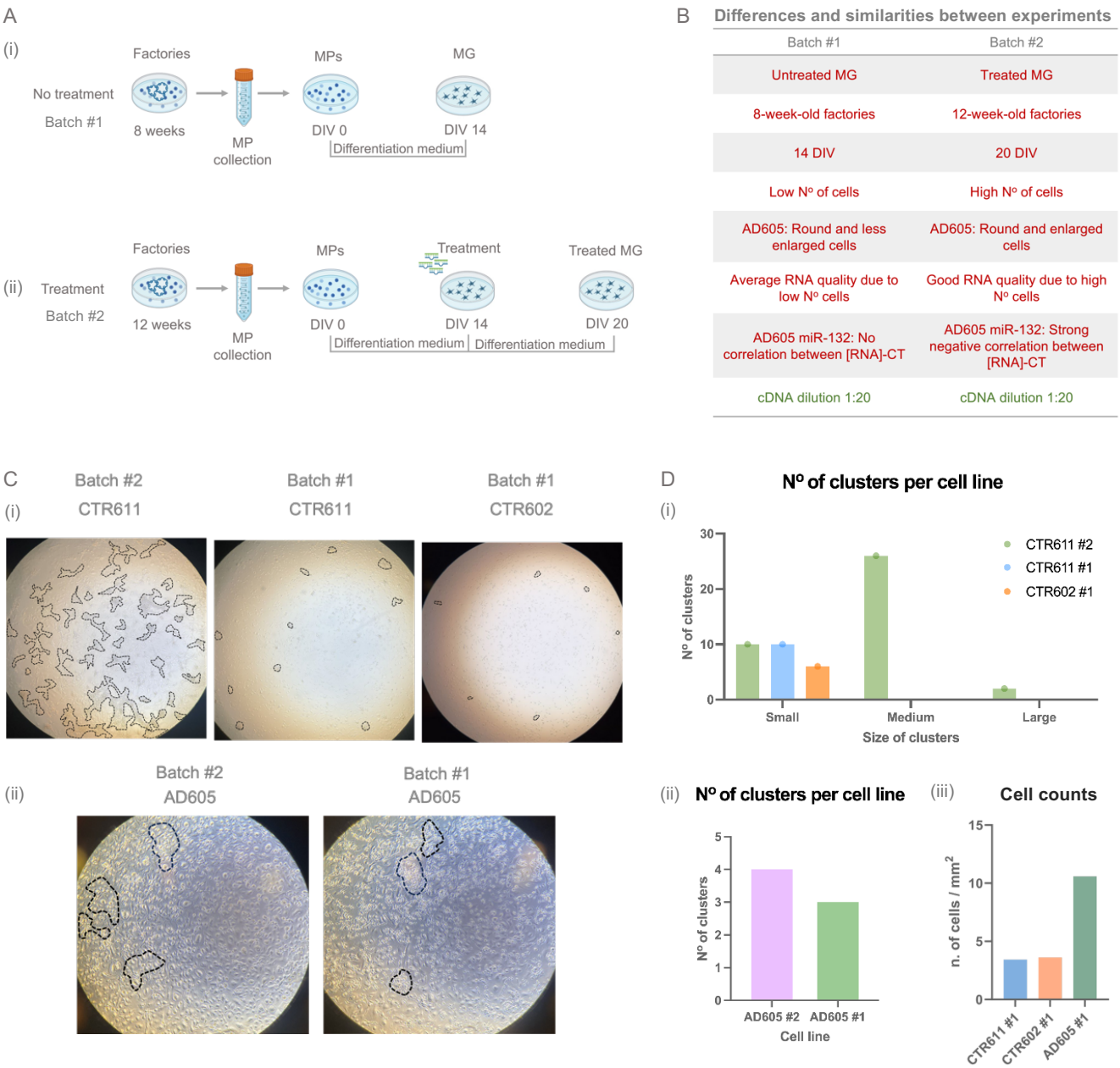


Figure S2. **Distinct experimental design between batch #1 and batch #2 experiments.**
A. Schematic representation showing the differences in the experimental design between batch #1 (i) and batch #2 (ii) experiments. B. Table indicating the differences and similarities between batch #1 (left) and batch #2 (right) experiments. C. Phone captured images of the different cell lines at 5x (i) and 10x (ii) magnification using a light microscope. The dashed lines indicated the cluster of cells observed in each line. D. Bar plots showing the number of clusters observed in the CTR (i) and AD (ii) cell lines. (iii) iPSC-derived microglial cells quantified as the number of cells per mm^2. Quantification of microglial cells was executed in confocal images with Fiji (ImageJ) using the StarDist plugin. CTR, control; AD, Alzheimer's disease.

8.3 Result outcome may be influenced by experiment execution

8.3.1 Evaluation of RNA quality

To better evaluate the quality of our results, we conducted a series of correlation analyses to determine whether experiment execution could have affected the outcome of the results and potentially explain the discrepancies between the two batches. First, we performed analyses to evaluate the quality of the extracted RNA, as indicated by the 260/230 and 260/280 ratio. Not surprisingly, we found that higher RNA concentration correlates with better RNA quality, whereas low RNA concentration is associated with poor RNA quality (Figure S3A). This, together with the previous observations on cell quantity, supports the notion that the RNA extraction procedure was performed correctly, and the poor RNA quality is likely attributed to other factors, such as contaminants including phenol. It is important to note that the observed poor RNA quality cannot be entirely ruled out as a potential contributing factor to the observed discrepancies between the two experiments, as it has been shown that contaminants can have an impact on gene expression (Carvalhais et al., 2013).

8.3.2 Correlation analyses between RNA concentration and threshold cycle (CT) in qPCR

To determine whether these discrepancies were a result of poor experiment execution, we next performed correlation analyses between the RNA concentration of the different samples and the CT values of miR-132, as well as the CT values of the combination of U6 and RnU5G housekeepers that were utilized for normalization in the analysis (Figure S3B). Given that both experiments started with the same RNA concentration and the same dilution was performed, it is not expected to observe any significant correlation between the RNA concentration and the CT values of any gene of interest. The identical starting conditions and dilution steps should result in consistent and comparable RNA concentrations across all samples, minimizing any potential correlations between the RNA concentration and the gene expression levels.

As expected, no correlation was observed between the RNA concentration and the CT values of miR-132 or the combination of U6 and RnU5G in batch #1 samples, across all lines (Figure S3Bi, iii). However, a strong negative correlation was observed between the RNA concentration and the CT values of both miR-132 and the combination of U6 and RnU5G for the AD605 line in batch #2, indicating either faulty dilution or an incomplete reverse transcriptase reaction (Figure S3Bii, iv). Considering that the AD605 line displayed a significant downregulation in batch #2 but not in batch #1, it is possible that the observed strong negative correlation between

the RNA concentration and the CT values, could potentially be a contributing factor to the inconsistent results.

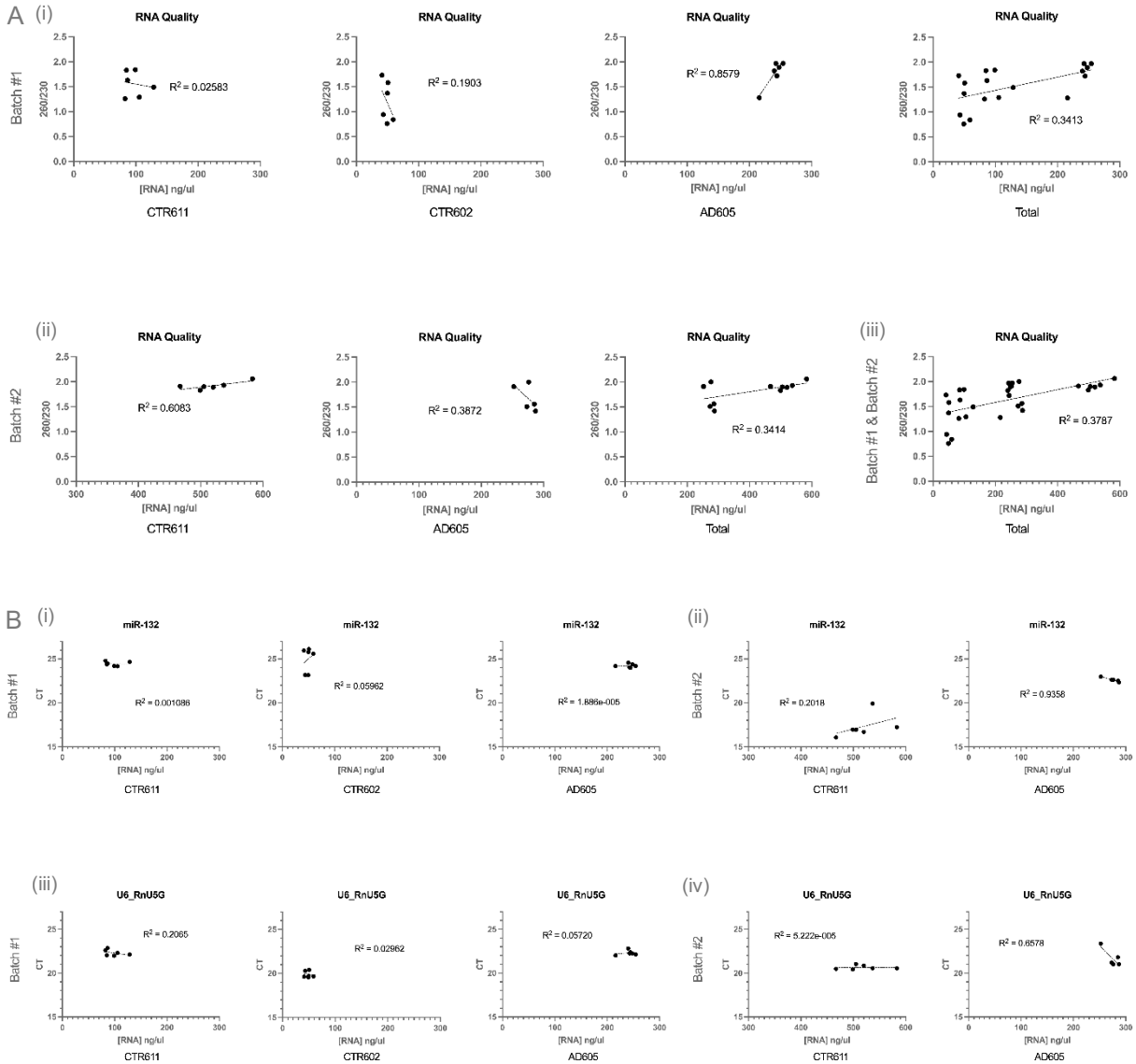


Figure S3. **Correlation analyses in batch #1 and batch #2 experiments.**
A. Scatterplots showing the correlation between RNA concentration and 260/230 ratio in batch #1(i), batch #2 (ii) and batch #1 and batch #2 (iii) experiments. Pearson's correlation (two-tailed, 95% confidence interval) and simple linear regression of correlation were performed. The R squared is provided within the plots. N = 5 – 6 technical replicates. **B**. Scatterplots showing the correlation between RNA concentration and CTs of miR-132 (i, ii) and U6 and RnU5G (iii, iv) in batch #1(i, iii) and batch #2 (ii, iv) experiments. Pearson's correlation (two-tailed, 95% confidence interval) and simple linear regression of correlation were performed. The R squared is provided within the plots. N = 5 – 6 technical replicates CTR, control; AD, Alzheimer's disease; miR-132, microRNA.

Table S1. RNA concentration and quality for batch #1 samples

qPCR Batch #1		[RNA] ng/ul	RNA Quality 260/280	260/230
CTR611	A	82.5	2.12	1.26
	B	128.5	2.07	1.49
	C	84.8	2.12	1.83
	D	86.4	2.03	1.63
	E	99	2.08	1.84
	F	105.4	2.03	1.29
AD605	A	244.2	2.14	1.72
	B	242.6	2.14	1.97
	C	215.9	2.14	1.28
	D	247.8	2.1	1.89
	E	254.3	2.06	1.97
	F	239.9	2.1	1.82
CTR602	A	49,8	1,91	1,37
	B	50,5	1,76	1,58
	C	41	1,87	1,73
	D	59,1	1,86	0,84
	E	43	1,82	0,94
	F	49,3	1,82	0,76

Table S2. RNA concentration and quality for batch #2 samples

qPCR Batch #2		[RNA] ng/ul	RNA Quality 260/280	260/230
CTR611	A	467	2,04	1,91
	B	583	2,12	2,06
	C	537	2,12	1,93
	D	505	2,13	1,9
	E	499	2,09	1,83
	F	520	2,1	1,89
AD605	A	276	2,07	2
	B	287	2,08	1,42
	C	273	2,06	1,51
	D	252	2,05	1,91
	E	285	2,06	1,56

References

Abud, E. M., Ramirez, R. N., Martinez, E. S., Healy, L. M., Nguyen, C. H. H., Newman, S. A., Yeromin, A. V., Scarfone, V. M., Marsh, S. E., Fimbres, C., Caraway, C. A., Fote, G. M., Madany, A. M., Agrawal, A., Kayed, R., Gylys, K. H., Cahalan, M. D., Cummings, B. J., Antel, J. P., … Blurton-Jones, M. (2017). iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron*, *94*(2), 278-293.e9. https://doi.org/10.1016/j.neuron.2017.03.042

Aloi, M. S., Prater, K. E., Sánchez, R. E. A., Beck, A., Pathan, J. L., Davidson, S., Wilson, A., Keene, C. D., de la Iglesia, H., Jayadev, S., & Garden, G. A. (2023). Microglia specific deletion of miR-155 in Alzheimer's disease mouse models reduces amyloid-β pathology but causes hyperexcitability and seizures. *Journal of Neuroinflammation*, *20*(1). https://doi.org/10.1186/s12974-023-02745-6

Andrews, S. J., Renton, A. E., Fulton-Howard, B., Podlesny-Drabiniok, A., Marcora, E., & Goate, A. M. (2023). The complex genetic architecture of Alzheimer's disease: novel insights and future directions. *EBioMedicine*, *90*, 104511. https://doi.org/10.1016/j.ebiom.2023.104511

Bhatnagar, D., Ladhe, S., & Kumar, D. (2023). Discerning the Prospects of miRNAs as a Multi-Target Therapeutic and Diagnostic for Alzheimer's Disease. *Molecular Neurobiology*. https://doi.org/10.1007/s12035-023-03446-0

Borst, K., Dumas, A. A., & Prinz, M. (2021). Microglia: Immune and non-immune functions. In *Immunity* (Vol. 54, Issue 10, pp. 2194–2208). Cell Press. https://doi.org/10.1016/j.immuni.2021.09.014

Brownjohn, P. W., Smith, J., Solanki, R., Lohmann, E., Houlden, H., Hardy, J., Dietmann, S., & Livesey, F. J. (2018). Functional Studies of Missense TREM2 Mutations in Human

Stem Cell-Derived Microglia. *Stem Cell Reports*, *10*(4), 1294–1307. https://doi.org/10.1016/j.stemcr.2018.03.003

Bushman, D. M., Kaeser, G. E., Siddoway, B., Westra, J. W., Rivera, R. R., Rehen, S. K., Yung, Y. C., & Chun, J. (2015). Genomic mosaicism with increased amyloid precursor protein (APP) gene copy number in single neurons from sporadic Alzheimer's disease brains. *ELife*, *4*. https://doi.org/10.7554/eLife.05116

Butovsky, O., Jedrychowski, M. P., Moore, C. S., Cialic, R., Lanser, A. J., Gabriely, G., Koeglsperger, T., Dake, B., Wu, P. M., Doykan, C. E., Fanek, Z., Liu, L., Chen, Z., Rothstein, J. D., Ransohoff, R. M., Gygi, S. P., Antel, J. P., & Weiner, H. L. (2014). Identification of a unique TGF-β-dependent molecular and functional signature in microglia. *Nature Neuroscience*, *17*(1), 131–143. https://doi.org/10.1038/nn.3599

Carvalhais, V., Delgado-Rastrollo, M., Melo, L. D. R., & Cerca, N. (2013). Controlled RNA contamination and degradation and its impact on qPCR gene expression in S. epidermidis biofilms. *Journal of Microbiological Methods*, *95*(2), 195–200. https://doi.org/10.1016/j.mimet.2013.08.010

Chen, Y., & Colonna, M. (2021). Microglia in Alzheimer's disease at single-cell level. Are there common patterns in humans and mice? In *Journal of Experimental Medicine* (Vol. 218, Issue 9). Rockefeller University Press. https://doi.org/10.1084/jem.20202717

Colonna, M., & Butovsky, O. (2017). Microglia function in the central nervous system during health and neurodegeneration. In *Annual Review of Immunology* (Vol. 35, pp. 441–468). Annual Reviews Inc. https://doi.org/10.1146/annurev-immunol-051116-052358

Cummings, J. (2018). Lessons Learned from Alzheimer Disease: Clinical Trials with Negative Outcomes. In *Clinical and Translational Science* (Vol. 11, Issue 2, pp. 147–152). Blackwell Publishing Ltd. https://doi.org/10.1111/cts.12491

De Strooper, B., & Karran, E. (2016). The Cellular Phase of Alzheimer's Disease. In *Cell* (Vol. 164, Issue 4, pp. 603–615). Cell Press. https://doi.org/10.1016/j.cell.2015.12.056

Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., & Amit, I. (2018). Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. In *Cell* (Vol. 173, Issue 5, pp. 1073–1081). Cell Press. https://doi.org/10.1016/j.cell.2018.05.003

Douvaras, P., Sun, B., Wang, M., Kruglikov, I., Lallos, G., Zimmer, M., Terrenoire, C., Zhang, B., Gandy, S., Schadt, E., Freytes, D. O., Noggle, S., & Fossati, V. (2017). Directed Differentiation of Human Pluripotent Stem Cells to Microglia. *Stem Cell Reports*, *8*(6), 1516–1524. https://doi.org/10.1016/J.STEMCR.2017.04.023

Dräger, N. M., Sattler, S. M., Huang, C. T. L., Teter, O. M., Leng, K., Hashemi, S. H., Hong, J., Aviles, G., Clelland, C. D., Zhan, L., Udeochu, J. C., Kodama, L., Singleton, A. B., Nalls, M. A., Ichida, J., Ward, M. E., Faghri, F., Gan, L., & Kampmann, M. (2022). A CRISPRi/a platform in human iPSC-derived microglia uncovers regulators of disease states. *Nature Neuroscience*, *25*(9), 1149–1162. https://doi.org/10.1038/s41593-022-01131-4

El Fatimy, R., Li, S., Chen, Z., Mushannen, T., Gongala, S., Wei, Z., Balu, D. T., Rabinovsky, R., Cantlon, A., Elkhal, A., Selkoe, D. J., Sonntag, K. C., Walsh, D. M., & Krichevsky, A. M. (2018). MicroRNA-132 provides neuroprotection for tauopathies via multiple signaling pathways. *Acta Neuropathologica*, *136*(4), 537–555. https://doi.org/10.1007/s00401-018-1880-5

Farrer, L. A., Cupples, ; L Adrienne, Haines, J. L., Hyman, B., Kukull, W. A., Mayeux, R., Myers, R. H., Pericak-Vance, M. A., Risch, N., & Van Duijn, C. M. (1997). *Effects of Age, Sex, and Ethnicity on the Association Between Apolipoprotein E Genotype and Alzheimer Disease A Meta-analysis*. http://jama.jamanetwork.com/

Friedman, B. A., Srinivasan, K., Ayalon, G., Meilandt, W. J., Lin, H., Huntley, M. A., Cao, Y., Lee, S. H., Haddick, P. C. G., Ngu, H., Modrusan, Z., Larson, J. L., Kaminker, J. S., van der Brug, M. P., & Hansen, D. V. (2018). Diverse Brain Myeloid Expression Profiles

Reveal Distinct Microglial Activation States and Aspects of Alzheimer's Disease Not Evident in Mouse Models. *Cell Reports*, *22*(3), 832–847. https://doi.org/10.1016/j.celrep.2017.12.066

Galatro, T. F., Holtman, I. R., Lerario, A. M., Vainchtein, I. D., Brouwer, N., Sola, P. R., Veras, M. M., Pereira, T. F., Leite, R. E. P., Möller, T., Wes, P. D., Sogayar, M. C., Laman, J. D., Den Dunnen, W., Pasqualucci, C. A., Oba-Shinjo, S. M., Boddeke, E. W. G. M., Marie, S. K. N., & Eggen, B. J. L. (2017). Transcriptomic analysis of purified human cortical microglia reveals age-associated changes. *Nature Neuroscience*, *20*(8), 1162–1171. https://doi.org/10.1038/nn.4597

Gaugler, J., James, B., Johnson, T., Scholz, K., & Weuve, J. (2016). 2016 Alzheimer's disease facts and figures. *Alzheimer's and Dementia*, *12*(4), 459–509. https://doi.org/10.1016/j.jalz.2016.03.001

Geirsdottir, L., David, E., Keren-Shaul, H., Weiner, A., Bohlen, S. C., Neuber, J., Balic, A., Giladi, A., Sheban, F., Dutertre, C. A., Pfeifle, C., Peri, F., Raffo-Romero, A., Vizioli, J., Matiasek, K., Scheiwe, C., Meckel, S., Mätz-Rensing, K., van der Meer, F., … Prinz, M. (2019). Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program. *Cell*, *179*(7), 1609-1622.e16. https://doi.org/10.1016/j.cell.2019.11.010

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M. F., Conway, S. J., Ng, L. G., Stanley, E. R., Samokhvalov, I. M., & Merad, M. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*, *330*(6005), 841–845. https://doi.org/10.1126/science.1194637

Gosselin, D., Skola, D., Coufal, N. G., Holtman, I. R., Schlachetzki, J. C. M., Sajti, E., Jaeger, B. N., O'Connor, C., Fitzpatrick, C., Pasillas, M. P., Pena, M., Adair, A., Gonda, D. D., Levy, M. L., Ransohoff, R. M., Gage, F. H., & Glass, C. K. (2017). An environment-dependent transcriptional network specifies human microglia identity. *Science*, *356*(6344), 1248–1259. https://doi.org/10.1126/science.aal3222

Grabert, K., Michoel, T., Karavolos, M. H., Clohisey, S., Kenneth Baillie, J., Stevens, M. P., Freeman, T. C., Summers, K. M., & McColl, B. W. (2016). Microglial brain regionâ 'dependent diversity and selective regional sensitivities to aging. *Nature Neuroscience*, *19*(3), 504–516. https://doi.org/10.1038/nn.4222

Gumbs, S. B. H., Kübler, R., Gharu, L., Schipper, P. J., Borst, A. L., Snijders, G. J. L. J., Ormel, P. R., van Berlekom, A. B., Wensing, A. M. J., de Witte, L. D., & Nijhuis, M. (2022). Human microglial models to study HIV infection and neuropathogenesis: a literature overview and comparative analyses. *Journal of NeuroVirology*, *28*(1), 64–91. https://doi.org/10.1007/s13365-021-01049-w

Haenseler, W., Sansom, S. N., Buchrieser, J., Newey, S. E., Moore, C. S., Nicholls, F. J., Chintawar, S., Schnell, C., Antel, J. P., Allen, N. D., Cader, M. Z., Wade-Martins, R., James, W. S., & Cowley, S. A. (2017). A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. *Stem Cell Reports*, *8*(6), 1727–1742. https://doi.org/10.1016/J.STEMCR.2017.05.017

Hansen, D. V., Hanson, J. E., & Sheng, M. (2018). Microglia in Alzheimer's disease. In *Journal of Cell Biology* (Vol. 217, Issue 2, pp. 459–472). Rockefeller University Press. https://doi.org/10.1083/jcb.201709069

Hanzel, C. E., Pichet-Binette, A., Pimentel, L. S. B., Iulita, M. F., Allard, S., Ducatenzeiler, A., Do Carmo, S., & Cuello, A. C. (2014). Neuronal driven pre-plaque inflammation in a transgenic rat model of Alzheimer's disease. *Neurobiology of Aging*, *35*(10), 2249–2262. https://doi.org/10.1016/j.neurobiolaging.2014.03.026

Hasselmann, J., & Blurton-Jones, M. (2020). Human iPSC-derived microglia: A growing toolset to study the brain's innate immune cells. *GLIA*, *68*(4), 721–739. https://doi.org/10.1002/glia.23781

Heppner, F. L., Ransohoff, R. M., & Becher, B. (2015). Immune attack: The role of inflammation in Alzheimer disease. In *Nature Reviews Neuroscience* (Vol. 16, Issue 6, pp. 358–372). Nature Publishing Group. https://doi.org/10.1038/nrn3880

Hickman, S., Izzy, S., Sen, P., Morsett, L., & El Khoury, J. (2018). Microglia in neurodegeneration. In *Nature Neuroscience* (Vol. 21, Issue 10, pp. 1359–1369). Nature Publishing Group. https://doi.org/10.1038/s41593-018-0242-x

Huang, K. L., Marcora, E., Pimenova, A. A., Di Narzo, A. F., Kapoor, M., Jin, S. C., Harari, O., Bertelsen, S., Fairfax, B. P., Czajkowski, J., Chouraki, V., Grenier-Boley, B., Bellenguez, C., Deming, Y., McKenzie, A., Raj, T., Renton, A. E., Budde, J., Smith, A., … Goate, A. M. (2017). A common haplotype lowers PU.1 expression in myeloid cells and delays onset of Alzheimer's disease. *Nature Neuroscience*, *20*(8), 1052–1061. https://doi.org/10.1038/nn.4587

Iovino, M., Agathou, S., González-Rueda, A., Del Castillo Velasco-Herrera, M., Borroni, B., Alberici, A., Lynch, T., O'Dowd, S., Geti, I., Gaffney, D., Vallier, L., Paulsen, O., Káradóttir, R. T., & Spillantini, M. G. (2015). Early maturation and distinct tau pathology in induced pluripotent stem cell-derived neurons from patients with MAPT mutations. *Brain*, *138*(11), 3345–3359. https://doi.org/10.1093/brain/awv222

Jeong, S. (2017). Molecular and cellular basis of neurodegeneration in alzheimer's disease. In *Molecules and Cells* (Vol. 40, Issue 9, pp. 613–620). Korean Society for Molecular and Cellular Biology. https://doi.org/10.14348/molcells.2017.0096

Jonsson, T., Stefansson, H., Steinberg, S., Jonsdottir, I., Jonsson, P. V., Snaedal, J., Bjornsson, S., Huttenlocher, J., Levey, A. I., Lah, J. J., Rujescu, D., Hampel, H., Giegling, I., Andreassen, O. A., Engedal, K., Ulstein, I., Djurovic, S., Ibrahim-Verbaas, C., Hofman, A., … Stefansson, K. (2013). Variant of TREM2 Associated with the Risk of Alzheimer's Disease . *New England Journal of Medicine*, *368*(2), 107–116. https://doi.org/10.1056/nejmoa1211103

Jurga, A. M., Paleczna, M., & Kuter, K. Z. (2020). Overview of General and Discriminating Markers of Differential Microglia Phenotypes. *Frontiers in Cellular Neuroscience*, *14*. https://doi.org/10.3389/fncel.2020.00198

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., David, E., Baruch, K., Lara-Astaiso, D., Toth, B., Itzkovitz, S., Colonna, M., Schwartz, M., & Amit, I. (2017). A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell*, *169*(7), 1276-1290.e17. https://doi.org/10.1016/j.cell.2017.05.018

Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E. G., Wieghofer, P., Heinrich, A., Riemke, P., Hölscher, C., Müller, D. N., Luckow, B., Brocker, T., Debowski, K., Fritz, G., Opdenakker, G., Diefenbach, A., Biber, K., Heikenwalder, M., … Prinz, M. (2013). Microglia emerge from erythromyeloid precursors via Pu.1-and Irf8-dependent pathways. *Nature Neuroscience*, *16*(3), 273–280. https://doi.org/10.1038/nn.3318

Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., El Fatimy, R., Beckers, L., O'Loughlin, E., Xu, Y., Fanek, Z., Greco, D. J., Smith, S. T., Tweet, G., Humulock, Z., Zrzavy, T., Conde-Sanroman, P., Gacias, M., Weng, Z., Chen, H., … Butovsky, O. (2017). The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity*, *47*(3), 566-581.e9. https://doi.org/10.1016/j.immuni.2017.08.008

Lambert, J. C., Ibrahim-Verbaas, C. A., Harold, D., Naj, A. C., Sims, R., Bellenguez, C., Jun, G., DeStefano, A. L., Bis, J. C., Beecham, G. W., Grenier-Boley, B., Russo, G., Thornton-Wells, T. A., Jones, N., Smith, A. V., Chouraki, V., Thomas, C., Ikram, M. A., Zelenika, D., … Seshadri, S. (2013). Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nature Genetics*, *45*(12), 1452–1458. https://doi.org/10.1038/ng.2802

Lambert, J. C., Ramirez, A., Grenier-Boley, B., & Bellenguez, C. (2023). Step by step: towards a better understanding of the genetic architecture of Alzheimer's disease. In *Molecular Psychiatry*. Springer Nature. https://doi.org/10.1038/s41380-023-02076-1

Lane, C. A., Hardy, J., & Schott, J. M. (2018). Alzheimer's disease. In *European Journal of Neurology* (Vol. 25, Issue 1, pp. 59–70). Blackwell Publishing Ltd. https://doi.org/10.1111/ene.13439

Lau, P., Bossers, K., Janky, R., Salta, E., Frigerio, C. S., Barbash, S., Rothman, R., Sierksma, A. S. R., Thathiah, A., Greenberg, D., Papadopoulou, A. S., Achsel, T., Ayoubi, T., Soreq, H., Verhaagen, J., Swaab, D. F., Aerts, S., & De Strooper, B. (2013). Alteration of the microRNA network during the progression of Alzheimer's disease. *EMBO Molecular Medicine*, *5*(10), 1613–1634. https://doi.org/10.1002/emmm.201201974

Laurijssens, B., Aujard, F., & Rahman, A. (2013). Animal models of Alzheimer's disease and drug development. In *Drug Discovery Today: Technologies* (Vol. 10, Issue 3). https://doi.org/10.1016/j.ddtec.2012.04.001

Leng, F., & Edison, P. (2021). Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? In *Nature Reviews Neurology* (Vol. 17, Issue 3, pp. 157–172). Nature Research. https://doi.org/10.1038/s41582-020-00435-y

Lin, Y. T., Seo, J., Gao, F., Feldman, H. M., Wen, H. L., Penney, J., Cam, H. P., Gjoneska, E., Raja, W. K., Cheng, J., Rueda, R., Kritskiy, O., Abdurrob, F., Peng, Z., Milo, B., Yu, C. J., Elmsaouri, S., Dey, D., Ko, T., … Tsai, L. H. (2018). APOE4 Causes Widespread Molecular and Cellular Alterations Associated with Alzheimer's Disease Phenotypes in Human iPSC-Derived Brain Cell Types. *Neuron*, *98*(6), 1141-1154.e7. https://doi.org/10.1016/j.neuron.2018.05.008

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Long, J. M., & Holtzman, D. M. (2019). Alzheimer Disease: An Update on Pathobiology and Treatment Strategies. In *Cell* (Vol. 179, Issue 2, pp. 312–339). Cell Press. https://doi.org/10.1016/j.cell.2019.09.001

Mancuso, R., Van Den Daele, J., Fattorelli, N., Wolfs, L., Balusu, S., Burton, O., Liston, A., Sierksma, A., Fourne, Y., Poovathingal, S., Arranz-Mendiguren, A., Sala Frigerio, C., Claes, C., Serneels, L., Theys, T., Perry, V. H., Verfaillie, C., Fiers, M., & De Strooper, B. (2019). Stem-cell-derived human microglia transplanted in mouse brain to study human disease. *Nature Neuroscience*, *22*(12), 2111–2116. https://doi.org/10.1038/s41593-019-0525-x

Masuda, T., Sankowski, R., Staszewski, O., Böttcher, C., Amann, L., Sagar, Scheiwe, C., Nessler, S., Kunz, P., van Loo, G., Coenen, V. A., Reinacher, P. C., Michel, A., Sure, U., Gold, R., Grün, D., Priller, J., Stadelmann, C., & Prinz, M. (2019). Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature*, *566*(7744), 388–392. https://doi.org/10.1038/s41586-019-0924-x

Mathys, H., Adaikkan, C., Gao, F., Young, J. Z., Manet, E., Hemberg, M., De Jager, P. L., Ransohoff, R. M., Regev, A., & Tsai, L. H. (2017). Temporal Tracking of Microglia Activation in Neurodegeneration at Single-Cell Resolution. *Cell Reports*, *21*(2), 366–380. https://doi.org/10.1016/j.celrep.2017.09.039

Moser, V. A., Workman, M. J., Hurwitz, S. J., Lipman, R. M., Pike, C. J., & Svendsen, C. N. (2021). Microglial transcription profiles in mouse and human are driven by APOE4 and sex. *IScience*, *24*(11). https://doi.org/10.1016/j.isci.2021.103238

Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., Bakiasi, G., Tsai, L. H., Aubourg, P., Ransohoff, R. M., & Jaenisch, R. (2016). Efficient derivation of microglia-like cells from human pluripotent stem cells. *Nature Medicine*, *22*(11), 1358–1367. https://doi.org/10.1038/nm.4189

Mungenast, A. E., Siegert, S., & Tsai, L. H. (2016). Modeling Alzheimer's disease with human induced pluripotent stem (iPS) cells. In *Molecular and Cellular Neuroscience* (Vol. 73, pp. 13–31). Academic Press Inc. https://doi.org/10.1016/j.mcn.2015.11.010

Okello, A., Edison, M. P., Archer, M. H., Turkheimer, M. F., Kennedy, J., Bullock, M. R., Walker, Mrcp. Z., Kennedy, A., Fox, N., Rossor, M., & Brooks, Ds. D. (2009). *Microglial activation and amyloid deposition in mild cognitive impairment A PET study*. www.neurology.org

Orre, M., Kamphuis, W., Osborn, L. M., Jansen, A. H. P., Kooijman, L., Bossers, K., & Hol, E. M. (2014). Isolation of glia from Alzheimer's mice reveals inflammation anddysfunction. *Neurobiology of Aging*, *35*(12), 2746–2760. https://doi.org/10.1016/j.neurobiolaging.2014.06.004

Pandya, H., Shen, M. J., Ichikawa, D. M., Sedlock, A. B., Choi, Y., Johnson, K. R., Kim, G., Brown, M. A., Elkahloun, A. G., Maric, D., Sweeney, C. L., Gossa, S., Malech, H. L., McGavern, D. B., & Park, J. K. (2017). Differentiation of human and murine induced pluripotent stem cells to microglia-like cells. *Nature Neuroscience*, *20*(5), 753–759. https://doi.org/10.1038/nn.4534

Parakalan, R., Jiang, B., Nimmi, B., Janani, M., Jayapal, M., Lu, J., Tay, S. S. W., Ling, E. A., & Dheen, S. T. (2012). Transcriptome analysis of amoeboid and ramified microglia isolated from the corpus callosum of rat brain. *BMC Neuroscience*, *13*(1). https://doi.org/10.1186/1471-2202-13-64

Reich, M., Paris, I., Ebeling, M., Dahm, N., Schweitzer, C., Reinhardt, D., Schmucki, R., Prasad, M., Köchl, F., Leist, M., Cowley, S. A., Zhang, J. D., Patsch, C., Gutbier, S., & Britschgi, M. (2021). Alzheimer's Risk Gene TREM2 Determines Functional Properties of New Type of Human iPSC-Derived Microglia. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.617860

Reiman, E. M., Arboleda-Velasquez, J. F., Quiroz, Y. T., Huentelman, M. J., Beach, T. G., Caselli, R. J., Chen, Y., Su, Y., Myers, A. J., Hardy, J., Paul Vonsattel, J., Younkin, S. G., Bennett, D. A., De Jager, P. L., Larson, E. B., Crane, P. K., Keene, C. D., Kamboh, M. I., Kofler, J. K., … Zhao, Y. (2020). Exceptionally low likelihood of Alzheimer's dementia in APOE2 homozygotes from a 5,000-person neuropathological study. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-019-14279-8

Riemens, R. J. M., Kenis, G., & van den Beucken, T. (2020). Human-induced pluripotent stem cells as a model for studying sporadic Alzheimer's disease. In *Neurobiology of Learning and Memory* (Vol. 175). Academic Press Inc. https://doi.org/10.1016/j.nlm.2020.107318

Salta, E., & De Strooper, B. (2017). MicroRNA-132: A key noncoding RNA operating in the cellular phase of Alzheimer's disease. *FASEB Journal*, *31*(2), 424–433. https://doi.org/10.1096/fj.201601308

Sankowski, R., Böttcher, C., Masuda, T., Geirsdottir, L., Sagar, Sindram, E., Seredenina, T., Muhs, A., Scheiwe, C., Shah, M. J., Heiland, D. H., Schnell, O., Grün, D., Priller, J., & Prinz, M. (2019). Mapping microglia states in the human brain through the integration of high-dimensional techniques. *Nature Neuroscience*, *22*(12), 2098–2110. https://doi.org/10.1038/s41593-019-0532-y

Schafer, S. T., Mansour, A. A. F., Schlachetzki, J. C. M., Pena, M., Ghassemzadeh, S., Mitchell, L., Mar, A., Quang, D., Stumpf, S., Ortiz, I. S., Lana, A. J., Baek, C., Zaghal, R., Glass, C. K., Nimmerjahn, A., & Gage, F. H. (2023). An in vivo neuroimmune organoid model to study human microglia phenotypes. *Cell*, *186*(10), 2111-2126.e20. https://doi.org/10.1016/j.cell.2023.04.022

Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S. E. W., Pollard, J. W., Frampton, J., Liu, K. J., & Geissmann, F. (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*, *336*(6077), 86–90.

Schwabenland, M., Brück, W., Priller, J., Stadelmann, C., Lassmann, H., & Prinz, M. (2021). Analyzing microglial phenotypes across neuropathologies: a practical guide. In *Acta Neuropathologica* (Vol. 142, Issue 6, pp. 923–936). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00401-021-02370-8

Sims, R., Van Der Lee, S. J., Naj, A. C., Bellenguez, C., Badarinarayan, N., Jakobsdottir, J., Kunkle, B. W., Boland, A., Raybould, R., Bis, J. C., Martin, E. R., Grenier-Boley, B., Heilmann-Heimbach, S., Chouraki, V., Kuzma, A. B., Sleegers, K., Vronskaya, M., Ruiz, A., Graham, R. R., … Schellenberg, G. D. (2017). Rare coding variants in PLCG2, ABI3, and TREM2 implicate microglial-mediated innate immunity in Alzheimer's disease. *Nature Genetics*, *49*(9), 1373–1384. https://doi.org/10.1038/ng.3916

Sposito, T., Preza, E., Mahoney, C. J., Setó-Salvia, N., Ryan, N. S., Morris, H. R., Arber, C., Devine, M. J., Houlden, H., Warner, T. T., Bushell, T. J., Zagnoni, M., Kunath, T., Livesey, F. J., Fox, N. C., Rossor, M. N., Hardy, J., & Wray, S. (2015). Developmental regulation of tau splicing is disrupted in stem cell-derived neurons from frontotemporal dementia patients with the 10 + 16 splice-site mutation in MAPT. *Human Molecular Genetics*, *24*(18), 5260–5269. https://doi.org/10.1093/hmg/ddv246

Srinivasan, K., Friedman, B. A., Larson, J. L., Lauffer, B. E., Goldstein, L. D., Appling, L. L., Borneo, J., Poon, C., Ho, T., Cai, F., Steiner, P., Van Der Brug, M. P., Modrusan, Z., Kaminker, J. S., & Hansen, D. V. (2016). Untangling the brain's neuroinflammatory and neurodegenerative transcriptional responses. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms11295

Tábuas-Pereira, M., Santana, I., Guerreiro, R., & Brás, J. (2020). Alzheimer's Disease Genetics: Review of Novel Loci Associated with Disease. *Current Genetic Medicine Reports*, *8*(1), 1–16. https://doi.org/10.1007/s40142-020-00182-y

Takahashi, K., & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*, *126*(4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Talwar, P., Sinha, J., Grover, S., Rawat, C., Kushwaha, S., Agarwal, R., Taneja, V., & Kukreti, R. (2016). Dissecting Complex and Multifactorial Nature of Alzheimer's Disease Pathogenesis: a Clinical, Genomic, and Systems Biology Perspective. In *Molecular Neurobiology* (Vol. 53, Issue 7, pp. 4833–4864). Humana Press Inc. https://doi.org/10.1007/s12035-015-9390-0

TCW, J., Qian, L., Pipalia, N. H., Chao, M. J., Liang, S. A., Shi, Y., Jain, B. R., Bertelsen, S. E., Kapoor, M., Marcora, E., Sikora, E., Andrews, E. J., Martini, A. C., Karch, C. M., Head, E., Holtzman, D. M., Zhang, B., Wang, M., Maxfield, F. R., … Goate, A. M. (2022). Cholesterol and matrisome pathways dysregulated in astrocytes and microglia. *Cell*, *185*(13), 2213-2233.e25. https://doi.org/10.1016/j.cell.2022.05.017

Timmerman, R., Burm, S. M., & Bajramovic, J. J. (2018). An overview of in vitro methods to study microglia. In *Frontiers in Cellular Neuroscience* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fncel.2018.00242

Villegas-Llerena, C., Phillips, A., Reitboeck, P. G., Hardy, J., & Pocock, J. M. (2016). Microglial genes regulating neuroinflammation in the progression of Alzheimer's disease. In *Current Opinion in Neurobiology* (Vol. 36, pp. 74–81). Elsevier Ltd. https://doi.org/10.1016/j.conb.2015.10.004

Walgrave, H., Balusu, S., Snoeck, S., Vanden Eynden, E., Craessaerts, K., Thrupp, N., Wolfs, L., Horré, K., Fourne, Y., Ronisz, A., Silajdžić, E., Penning, A., Tosoni, G., Callaerts-Vegh, Z., D'Hooge, R., Thal, D. R., Zetterberg, H., Thuret, S., Fiers, M., … Salta, E. (2021). Restoring miR-132 expression rescues adult hippocampal neurogenesis and memory deficits in Alzheimer's disease. *Cell Stem Cell*, *28*(10), 1805-1821.e8. https://doi.org/10.1016/j.stem.2021.05.001

Walgrave, H., Penning, A., Tosoni, G., Snoeck, S., Davie, K., Davis, E., Wolfs, L., Sierksma, A., Mars, M., Bu, T., Thrupp, N., Zhou, L., Moechars, D., Mancuso, R., Fiers, M.,

Howden, A. J. M., De Strooper, B., & Salta, E. (2023). microRNA-132 regulates gene expression programs involved in microglial homeostasis. *IScience*, *26*(6), 106829. https://doi.org/10.1016/j.isci.2023.106829

Walgrave, H., Zhou, L., De Strooper, B., & Salta, E. (2021). The promise of microRNA-based therapies in Alzheimer's disease: challenges and perspectives. *Molecular Neurodegeneration*, *16*(1). https://doi.org/10.1186/s13024-021-00496-7

Wanet, A., Tacheny, A., Arnould, T., & Renard, P. (2012). MiR-212/132 expression and functions: Within and beyond the neuronal compartment. *Nucleic Acids Research*, *40*(11), 4742–4753. https://doi.org/10.1093/nar/gks151

Wang, Y., Ulland, T. K., Ulrich, J. D., Song, W., Tzaferis, J. A., Hole, J. T., Yuan, P., Mahan, T. E., Shi, Y., Gilfillan, S., Cella, M., Grutzendler, J., DeMattos, R. B., Cirrito, J. R., Holtzman, D. M., & Colonna, M. (2016). TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques. *Journal of Experimental Medicine*, *213*(5), 667–675. https://doi.org/10.1084/jem.20151948

World Health Organization. (2023, March 15). *Dementia*. https://www.who.int/news-room/fact-sheets/detail/dementia

Xu, M., Zhang, L., Liu, G., Jiang, N., Zhou, W., & Zhang, Y. (2019). Pathological Changes in Alzheimer's Disease Analyzed Using Induced Pluripotent Stem Cell-Derived Human Microglia-Like Cells. *Journal of Alzheimer's Disease*, *67*(1), 357–368. https://doi.org/10.3233/JAD-180722

Xu, R., Li, X., Boreland, A. J., Posyton, A., Kwan, K., Hart, R. P., & Jiang, P. (2020). Human iPSC-derived mature microglia retain their identity and functionally integrate in the chimeric mouse brain. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-15411-9

Young-Pearse, T. L., Lee, H., Hsieh, Y. C., Chou, V., & Selkoe, D. J. (2023). Moving beyond amyloid and tau to capture the biological heterogeneity of Alzheimer's disease. In *Trends in Neurosciences*. Elsevier Ltd. https://doi.org/10.1016/j.tins.2023.03.005

Yvanka de Soysa, T., Therrien, M., Walker, A. C., & Stevens, B. (2022). Redefining microglia states: Lessons and limits of human and mouse models to study microglia states in neurodegenerative diseases. In *Seminars in Immunology* (Vol. 60). Academic Press. https://doi.org/10.1016/j.smim.2022.101651

Zerbino, D. R., Achuthan, P., Akanni, W., Amode, M. R., Barrell, D., Bhai, J., Billis, K., Cummins, C., Gall, A., Girón, C. G., Gil, L., Gordon, L., Haggerty, L., Haskell, E., Hourlier, T., Izuogu, O. G., Janacek, S. H., Juettemann, T., To, J. K., … Flicek, P. (2018). Ensembl 2018. *Nucleic Acids Research*, *46*(D1), D754–D761. https://doi.org/10.1093/nar/gkx1098