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DOUTORAMENTO

BIOTECNOLOGIA MOLECULAR E CELULAR APLICADA ÀS CIÊNCIAS DA SAÚDE

Non-coding RNAs as regulators of Inflammation and Depression

João Paulo Heitor Brás

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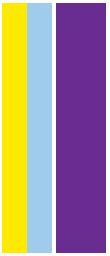
2021



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SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE FARMÁCIA

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João Paulo Heitor Brás

Tese de Doutoramento em Biotecnologia Molecular e Celular Aplicada às Ciências da Saúde

Porto, Julho 2021

João Paulo Heitor Brás

Non-coding RNAs as regulators of Inflammation and Depression

Tese de Candidatura ao grau de Doutor em Biotecnologia Molecular e Celular Aplicada às Ciências da Saúde, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto e Faculdade de Farmácia da Universidade do Porto

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À minha família,
ao meu avô.

Thesis publications

Complying with the terms of *nº 2, alínea a) do artigo 31.º do Decreto-Lei n.º230/2009*, the work developed in this doctoral thesis is published, under review or in preparation for publication in peer-reviewed scientific journals, as following detailed:

Article I

Book Chapter published

João P. Brás, Sara Pinto, Maria I. Almeida, Joana Prata, Orlando von Doellinger, Rui Coelho, Mário A Barbosa, Susana G. Santos. Peripheral Biomarkers of Inflammation in Depression: Evidence from Animal Models and Clinical Studies. Methods Mol Biol. 2019;2011:467-492. doi: 10.1007/978-1-4939-9554-7_28.
URL: https://link.springer.com/protocol/10.1007%2F978-1-4939-9554-7_28

Article II

Original research article published

João P. Brás, Joana Bravo, Jaime Freitas, Mário A. Barbosa, Susana G. Santos, Teresa Summavielle, Maria I. Almeida. TNF-alpha-induced microglia activation requires miR-342: impact on NF-κB signalling and neurotoxicity. Cell Death Dis. 2020 Jun 2;11(6):415. doi: 10.1038/s41419-020-2626-6.
URL: https://www.nature.com/articles/s41419-020-2626-6

Article III

Original research article under review

João P. Brás, Isabelle de Suduiraut, Olivia Zanoletti, Silvia Monari, Mandy Meijer, Jocelyn Grosse, Mário A. Barbosa, Susana G. Santos, Carmen Sandi, Maria I. Almeida. Stress-induced depressive-like behaviour in male rats is associated with microglial activation and inflammation dysregulation in the hippocampus in adulthood. *Under review in an international peer reviewed journal*

Article IV

Original research article in preparation

João P. Brás, Sara Pinto, Joana Prata, Orlando von Doellinger, Rui Coelho, Mário A. Barbosa, Maria I. Almeida, Susana G. Santos. A clinical study of inflammatory and miRNA mediators in depression. *In preparation*

Complementary publications in fields related to the thesis

Scientific articles:

1. **João P. Brás**, Andreia M. Silva, George A. Calin, Mário A. Barbosa, Susana G. Santos, Maria I. Almeida. miR-195 inhibits macrophages pro-inflammatory profile and impacts the crosstalk with smooth muscle cells. PLoS One. 2017 Nov 22;12(11):e0188530. doi: 10.1371/journal.pone.0188530.
URL: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0188530

2. Sara Gomes, Bartolomeo Bosco, Joana B. Loureiro, Helena Ramos, Liliana Raimundo, Joana Soares, Nair Nazareth, Valentina Barcherini, Lucília Domingues, Carla Oliveira, Alessandra Bisio, Silvano Piazza, Matthias R. Bauer, **João P. Brás**, Maria I. Almeida, Célia Gomes, Flávio Reis, Alan R. Fersht, Alberto Inga, Maria M.M. Santos, Lucília Saraiva. SLMP53-2 Restores Wild-Type-Like Function to Mutant p53 through Hsp70: Promising Activity in Hepatocellular Carcinoma. Cancers (Basel). 2019 Aug 10;11(8):1151. doi: 10.3390/cancers11081151-
URL: https://www.mdpi.com/2072-6694/11/8/1151

3. Sara R. Moura, **João P. Brás**, Jaime Freitas, Hugo Osório, Mário A. Barbosa, Susana G. Santos, Maria I. Almeida. miR-99a in bone homeostasis: Regulating osteogenic lineage commitment and osteoclast differentiation. Bone. 2020 May;134:115303. doi: 10.1016/j.bone.2020.115303.
URL:
https://www.sciencedirect.com/science/article/abs/pii/S8756328220300831?via%3Dihub

4. Mafalda Bessa-Gonçalves, Andreia M. Silva, **João P. Brás**, Heike Helmholz, Bérengère Luthringer-Feyerabend, Regine Willumeit-Römer, Mário A. Barbosa MA, Susana G. Santos. Fibrinogen and magnesium combination biomaterials modulate macrophage phenotype, NF-κB signalling and crosstalk with mesenchymal stem/stromal cells. Acta Biomater. 2020 Sep 15;114:471-484. doi: 10.1016/j.actbio.2020.07.028.
URL: https://www.sciencedirect.com/science/article/pii/S1742706120304141

Financial support

This work was funded by project NORTE-01-0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), and by grants from the Swiss National Science Foundation (SNSF) [NCCR Synapsy: 51NF40-158776 and -185897] and ERA-NET (Biostress; SNSF No 31NE30_189061). João Brás was supported by FCT- Fundação para a Ciência e Tecnologia, through BiotechHealth PhD program fellowship (PD/BD/135490/2018).

Acknowledgments

Hoje é o dia da última etapa da viagem mais desafiante da minha vida. Foi uma viagem digna de um *Tour de France*. Houve etapas duras de montanha, outras que foram autênticos contrarrelógios. Houve inclusive alguns furos e quedas pelo meio. Ainda assim, consegui chegar ao fim. Realizado, orgulhoso, feliz e grato. Grato por poder ter contado com a melhor "equipa" do pelotão. Uma "equipa" composta por "atletas" de alta competição. Atletas que me fizeram perceber que por muito sucesso individual que possamos conseguir na vida, não fazemos nada sozinhos. Atletas que fizeram de mim melhor investigador e acima de tudo melhor pessoa. Foi de vós que bebi conhecimento, foi convosco que descobri coisas que jamais sonhei existirem, foi em vocês que me refugiei depois das quedas. E a vocês devo este agradecimento:

A ti **Inês**, a minha mãe na Ciência, obrigado teres sido a minha "guiding light" durante esta viagem. Sabes da importância que tiveste neste projeto, no meu crescimento, nas oportunidades que me foste criando para aprender mais e mais. O "menino" João que a Janeiro de 2015 no antigo INEB foi a uma entrevista com as Doutoras Susana Santos e Inês Almeida, cresceu bastante desde então. Ainda me lembro de no primeiro dia em que me apresentei no laboratório do ICBAS, tu pediste-me de imediato por não te tratar por "Professora": "Chama-me só Inês, e trata-me por tu!". Foi giro... porque depois disso puseste-me a pipetar água! E se valeu a pena... "moldaste-me", "construíste-me" como uma casa... como se fosse a tua casa. Pedra a pedra, foste-me ensinando, dando espaço para tentar, errar, e voltar a tentar. Assim, ganhei confiança, autonomia, ter gosto em fazer e em fazer bem. Obrigado por manteres a exigência lá em cima. Por cobrares... mesmo que por vezes eu pensasse cá para mim: "ela está louca, não vou conseguir para fazer isto a tempo!". A verdade é que conseguia! Aprendi a gerir melhor o tempo, a fazer sacrifícios, quando têm de ser feitos. Porque havia um compromisso, mas acima de tudo, por mim... porque percebi que o que o querias, e sempre quiseste, era o melhor para mim. Obrigado por isso e por tanto.

A si **Susana**, muito obrigado pelo apoio constante e por acreditar em mim ao longo de todos estes anos. A Susana tem um dom que talvez desconheça. Sabe aquelas alturas em que estamos a bater com a cabeça na parede, ou porque não sabemos como interpretar uns resultados ou porque algo não está a funcionar e não conseguimos perceber porquê? A Susana tem o dom de dar as respostas a todas estas perguntas! É um porto seguro, de onde saímos sempre com novas ideias e ainda mais motivados. Obrigado por ser a incrível profissional que é. Por gostar de orientar, mais do que supervisionar. Por dar espaço aos

seus alunos, sem nunca os abandonar. Por se preocupar com os projetos que lidera, mas acima de tudo, por se preocupar e querer sempre o melhor para os seus “meninos”.

Ao **Prof. Mário**, obrigado por ter me acolhido no seu, no nosso grupo, durante os últimos 6 anos. Obrigado pela forma como sempre se preocupou em dar o maior contributo e atenção possíveis aos meus projetos, ainda que eles abrangessem áreas ligeiramente diferentes das linhas de investigação do grupo. A forma como fomenta o olhar crítico sobre tudo o que fazemos ou obtemos, é sem dúvida a maior lição que levo para vida. Foi e será sempre um privilégio aprender e trabalhar consigo.

To **Prof. Carmen** for the incredible availability and openness you showed, since the first moment, to collaborate and welcome me in your Lab. Without knowing a lot about me, you trusted my project, hosted me into your fantastic team, and always valued my comments on the projects that were being developed. Thank you for investing in me and for all the knowledge you have passed me. It was an intense and challenging year, with a pandemic in the middle, but which I will keep as one of the best experiences of my life.

Ao **Prof. Rui Coelho**, **Prof. Orlando von Doellinger**, **Dra. Joana Prata** e **Dra. Sara Pinto** pelo esforço e contributo por levar o projeto a bom porto, no constante recrutamento e monitorização de doentes.

À **Prof. Teresa Summavielle**, pelos constantes inputs que foi dando ao meu projeto e por sempre mostrar uma abertura sincera e genuína para colaborar e ajudar naquilo que eu precisava. À **Joana Bravo** por fazeres as honras na minha introdução ao mundo da microglia e pela disponibilidade e apoio fulcral que me deste numa fase tão embrionária do meu projeto de doutoramento. Ao **Prof. João Relvas** pela amabilidade com que me cedeu as células N9.

Às meninas do CCGEN, **Paula** e **Tânia**, e ao **Hugo Osório**, pela ajuda e conhecimento profundo de técnicas que foram cruciais para esta tese. À **Dalila** e ao **Ricardo**, pelo fantástico suporte que nos dão no dia-a-dia do laboratório.

Ao **Prof. Manuel Vilanova** e ao **Prof. Mário Barbosa**, na qualidade de Diretores do Programa Doutoral em Biotecnologia Molecular e Celular Aplicada às Ciências da Saúde, pelo valioso suporte institucional durante todo o doutoramento.

Ao fantástico grupo de alunos da **4ª edição do BiotechHealth**, **Rita**, **Andreia**, **Eunice**, **Filipe**, **Mafalda**, **Joana** e **José**. Obrigado pelo sentimento de partilha e entreajuda que sempre demonstraram. Criámos um grupo unido, amigo, que olhou sempre pelo outro. Isso mostra bem a essência de cada um vós. Malta, os *Internationals* vão mesmo ser doutores!

Aos meus colegas do MiNT. Aos que ainda continuam e aos que se cruzaram comigo nesta etapa, mas que já abandonaram o grupo. Quem passa por esta equipa sabe que mais do que relações profissionais, criam-se laços e amizades muito especiais. Muito obrigado **Andreia**, **Zé**, **Mafalda**, **Morena**, **Cláudia**, **Joana**, **Luísa**, **Sara**, **Jaime** e tantos outros, pelo apoio no laboratório, mas sobretudo por proporcionarem um ambiente tão especial no Lab 105.S1. **Um agradecimento especial** a ti **Andreia**, pela importância que tiveste no meu crescimento, por tudo aquilo que me ensinaste, por estares sempre disponível para ajudar. Tens sido e serás sempre a minha maior referência no que diz respeito à dedicação, ao rigor e à vontade de fazer bem, não por nós, mas por um bem maior. A ti **Zé**, pelos conselhos, pelas palavras de encorajamento, pelos ensinamentos, pelo teu contributo para este projeto. Seja pelas viagens que fizeste a Penafiel, pelas amostras que processaste, pelas gotas que apanhaste até às 4 da manhã... Esta tese tem também tem tua impressão digital. A ti **Mafalda**, minha companheira de viagem, minha amiga. Chegaste ao lab para desenvolver o teu projeto de doutoramento. Curiosamente durante as primeiras semanas, acho que nem sabia o teu nome. Só quando começámos a ter as primeiras aulas do programa doutoral é que interagimos. Mas percebe-se... Foi preciso quebrar o gelo, dado que és uma pessoa bastante introvertida, delicada e discreta (só que não!). Agora a sério, posso dizer que fomos o ponto de equilíbrio um do outro. Sempre que precisámos de ajuda, na bancada ou nos sofás, sabíamos que podíamos contar um com outro. E isso é bonito. É por isso que gosto muito de ti e sabes que tens aqui um amigo para vida. To you **Morena**, for being such a special person. You always have the best gestures or says to make my days happier. The truth is, no matter how disappointed or upset we might be with something, when we're together, I bet that we're going to end up laughing. That is what friends are made for. To rescue us when we're down, and to boost our confidence and positive energy. And I am lucky enough to have you as my friend. **Cláudia**, **Joana** e **Luísa** obrigado pelas conversas animadas, divertidas, que fizeram os meus dias mais felizes e mais fáceis de tantas maneiras. **Sara** e **Jaime**, obrigado por segurarem o barco, enquanto estive ausente, por estar fora ou durante as férias. O vosso contributo para este trabalho foi importantíssimo e agradeço-vos muito por isso.

To my colleagues at the LGC, especially **Olivia**, **Silvia**, **Isabelle**, **Mandy**, **Jocelyn**, **Clarissa**, **Ale**, **Elias** and **João**! In your own way, each one of you cared about me, and tried to make my life a little better, despite being so far apart from home. For over a year, you were my family, and as good family members, you were responsible for making this experience one of the most enriching and spectacular of my life. Thank you also to the BIOP Platform, especially Romain, for the help with my challenging imaging analysis.

Aos meus **pais**, **avós** e **tias** obrigado por tudo. Obrigado por darem tanto de vocês por tão pouco que vos posso dar em troca. Obrigado por estarem sempre ao meu lado nas escolhas que tenho feito ao longo da vida. Obrigado pelos valores que me incutiram. Sabemos o quão difíceis foram estes últimos anos. Mas estamos aqui, juntos, para continuarmos a ser felizes. Ainda que não percebam uma palavra do que está escrito daqui para a frente, eu sei vocês estão orgulhosos, apenas e só porque sabem que estou feliz. Esta tese é para vocês e para a estrelinha que nos ilumina todos os dias.

A ti **Cláudia**, obrigado por seres quem és. Por me conheceres tão bem, por compreenderes os meus silêncios, os meus olhares, os meus sorrisos, as minhas gargalhadas. Por não me cobrares as noites em que tenho de estar agarrado ao computador, as idas ao laboratório aos fins de semana, as frustrações que por vezes levo para casa. És a minha melhor amiga, minha confidente, minha companheira de viagens, de aventuras... és o meu porta-chaves! Juntos temos conseguido desafiar-nos e motivar-nos para fazer sempre mais e melhor. Para abraçar novas experiências e novos desafios. E assim vai continuar a ser, porque esta nossa viagem ainda agora está a começar...

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Abstract

Depression is a serious mood disorder affecting more than 264 million people of all ages worldwide (WHO, 2020). It is a leading cause of disability and a major contributor to the global burden of disease. Despite current anti-depressants being considered efficient in depression treatment, one third of patients does not respond to the first treatment regime, and for over 75% of patients have recurrent episodes of depressive disease. It is believed that the major barrier to effective treatments is inaccurate diagnosis.

Inflammation has been associated to depression symptomatology and treatment resistance. On one hand, a segment of depressive patients exhibits low-grade chronic inflammation with increased levels of inflammatory markers, such as tumour necrosis factor-α (TNF-α), that are worsened in treatment non-responders. On the other hand, the incidence of depression in patients with chronic inflammatory diseases is substantially higher. However, the molecular mechanisms underlying this interconnection are still unclear.

Non-coding RNAs, specifically microRNAs' (miRNAs) have unique expression patterns and the ability to modulate mRNA levels of a large number of target genes. This makes them a promising tool to understand disease biological mechanisms. Moreover, miRNAs have been found aberrantly expressed in several pathologies, increasing their potential as biomarkers.

In this PhD thesis, we hypothesized that specific miRNAs would be involved in the regulatory mechanisms underlying the association between inflammation and depressive traits, and that uncovering these may lead to novel diagnostic and therapeutic approaches. Thus, we specifically aimed to: (i) explore the role of inflammation-related miRNAs in microglia activation and in microglia-to-neurons communication; (ii) understand how stress impacts inflammation-associated miRNAs in the brain, *in vivo*, using a rat model of stress inductor of depressive-like behaviours; (iii) identify new biomarkers of depression by validating miRNA candidates in peripheral blood of depression patients.

In the first task, we specifically aimed to identify novel TNF-α-induced miRNAs and to dissect their roles in microglia activation, as well as to explore their impact on the cellular communication with neurons, *in vitro*. Stimulation of primary rat microglia with TNF-α led to an upregulation of pro-inflammatory markers *Nos2*, *Tnf, Il1b* and ph-NF-κB p65, and dysregulated the expression of several miRNAs. Of those, miR-342 was further validated and found to be significantly overexpressed. Gain- and loss-of-function *in vitro* assays and proteomic analysis were then used to dissect the role of miR-342 in microglia activation. Interestingly, miR-342 overexpression in N9 microglia cells was sufficient to activate the

NF-κB pathway by inhibiting BAG-1, leading to increased secretion of TNF-α and IL-1β. Conversely, miR-342 inhibition led to a strong decrease in the levels of these cytokines after TNF-α activation. Finally, co-cultures of microglia with hippocampal neurons, using a microfluidic system, were performed to understand the impact on neurotoxicity. Both TNF-α-stimulated and miR-342-overexpressing microglia drastically affected neuron viability. Remarkably, increased levels of nitrites were detected in the supernatants of these co-cultures. Globally, our findings indicate miR-342 as a crucial mediator of TNF-α-mediated microglia activation. These results are compiled in the scientific article Brás *et al.*, Cell Death and Disease (2020).

Next, in order to evaluate if miR-342 would actually be dysregulated and associated with depression and inflammation, we performed an *in vivo* rat model of early-life stress inductor of long-term depressive-like behaviours. Specifically, we explored how peripubertal stress (PPS) combined with differential corticosterone (CORT)-stress responsiveness (CSR) influences depressive-like behaviours and the expression of brain inflammatory markers, including miR-342, in adulthood. We found that high-CORT stress-responsive (H-CSR) male rats that underwent PPS exhibited increased depressive-like behaviours in adulthood. Also, animals exposed to PPS showed increased hippocampal TNF-α expression, which positively correlated with passive coping responses. In addition, PPS caused late effects on hippocampal microglia, particularly in H-CSR rats, with increased hippocampal IBA-1 expression and morphological alterations compatible with a higher degree of activation. Strikingly, H-CSR animals that underwent PPS exhibited increased expression of hippocampal miR-342, and its expression was positively correlated with TNF-α, microglial activation and passive coping responses. These results not only corroborated, *in vivo*, our previous findings, of a strong interplay between miR-342, TNF-α and microglial activation, but also showed, for the first time, an aberrant expression of miR-342 in the brain of rats exhibiting depressive-like behaviours. These results were compiled in a scientific article that is now under review in an international peer reviewed journal.

Finally, to explore the potential of selected inflammation-related miRNAs, particularly miR-342, as peripheral diagnostic markers of depression, a proof-of-concept clinical study was performed. Here, 40 healthy controls and 32 depression patients prior to treatment were included. The levels of inflammatory cytokines in plasma and their expression levels in PBMC were evaluated. Also, expression of miR-145, miR-146a, miR-155 and miR-342 in PBMCs, was measured. Interestingly, the studied cohort of depression patients was found to have a pro-inflammatory profile in plasma, with significantly higher levels of TNF-α and CCL2 compared with healthy controls. In PBMCs, TNF-α and IL-6 expression levels were significantly up and downregulated in depression patients, respectively. Moreover, miR-342 levels were found upregulated, while miR-146a and miR-155 were significantly

downregulated in PBMCs of depression patients. Of note, expression levels of miR-342 were positively correlated with TNF-α in PBMCs. Interestingly, when tested as a diagnostic panel, the use of miR-342, miR-146a, miR-155 in combination was the best classifier (AUC=0.842, CI=0.75-0.93), with potential to constitute a good diagnostic tool for depression. These results were compiled in a manuscript, that is being prepared for submission.

Overall, the work developed in this thesis contributed to further understand the interconnection between inflammation and depression, by exploring deeper how miRNAs regulate and appear dysregulated in both processes. Globally, the results presented and discussed here identify miR-342 as a potential diagnostic and therapeutic target particularly when inflammatory mediators, like TNF-α, are overexpressed. We described the mechanism by which it contributes to TNF-α driven microglia activation, and found it upregulated in the hippocampus of rats exhibiting depressive-like behaviours and increased neuroinflammation. Finally, miR-342 was also found upregulated in PBMCs of depression patients, and together with other two inflammatory miRNAs, miR-146a and miR-155, were shown to constitute a potential biomarker panel for depression. Future studies should investigate and validate these findings in larger cohorts of patients, establishing the potential of these and other miRNAs as specific diagnostic and treatment-response biomarkers for depression.

Resumo

A depressão é uma perturbação mental que afeta mais de 264 milhões de pessoas de todas as idades em todo o mundo (OMS, 2020). É uma das principais causas de incapacitação e um dos principais contribuintes para a carga global de doenças. Apesar dos antidepressivos atuais serem considerados eficazes no tratamento da depressão, um terço dos doentes não responde ao primeiro regime de tratamento e mais de 75% dos doentes apresentam episódios recorrentes de depressão. A principal barreira a tratamentos mais eficazes é o diagnóstico impreciso.

A inflamação tem sido associada à sintomatologia da depressão e à resistência ao tratamento. Por um lado, um segmento de doentes diagnosticados com depressão exibe inflamação crónica de baixo grau com níveis aumentados de marcadores inflamatórios, como o fator de necrose tumoral-α (TNF-α), que se agravam nos pacientes que não respondem ao tratamento. Por outro lado, a incidência de depressão em doentes com patologias inflamatórias crónicas é substancialmente maior. No entanto, os mecanismos moleculares subjacentes a esta interconexão ainda não são claros.

Os RNAs não codificantes, especificamente os microRNAs (miRNAs), têm padrões de expressão únicos e capacidade de modular os níveis de mRNA de um grande número de genes alvo. Estas características fazem deles ferramentas promissoras para entender os mecanismos biológicos de várias doenças. Para além disso, os níveis de alguns miRNAs aparecem desregulados em diversas patologias, o que aumenta o seu potencial como biomarcadores.

Nesta tese de doutoramento, formulamos a hipótese de que alguns miRNAs específicos poderiam estar envolvidos nos mecanismos subjacentes à associação entre a inflamação e a depressão, pelo que a sua descoberta pode levar a novas abordagens ao nível do diagnóstico e da terapêutica. Neste sentido, procurámos especificamente: (i) explorar o papel de miRNAs inflamatórios na ativação da microglia e na comunicação da microglia com os neurónios; (ii) compreender como o stress afeta os níveis dos miRNAs identificados, no cérebro, *in vivo*, usando um modelo animal de stress indutor de comportamentos-tipo depressivos; (iii) identificar novos biomarcadores da depressão, avaliando os níveis de vários miRNAs no sangue periférico de doentes diagnosticados com depressão.

Na primeira tarefa, tivemos como objetivo identificar novos miRNAs induzidos pelo TNF-α, dissecar o seu papel na ativação da microglia, bem como explorar o seu impacto na comunicação celular com os neurónios, *in vitro*. A estimulação de células da microglia de

rato com TNF-α induziu a sobre expressão de marcadores pró-inflamatórios como o *Nos2, Tnf, Il1b* e *ph-NF-κB p65,* assim como desregulação da expressão de vários miRNAs. Destes, o miR-342 foi posteriormente validado como estando significativamente sobre expresso. Depois, por forma a dissecar o papel do miR-342 na ativação da microglia, foram realizados ensaios *in vitro* de ganho e perda de função e uma análise proteómica para identificação de possíveis alvos. A sobre expressão do miR-342 em células N9 da microglia foi suficiente para ativar a via do NF-κB por inibição do BAG-1, levando ao aumento da secreção de TNF-α e de IL-1β. Por outro lado, a inibição do miR-342 levou a uma forte diminuição nos níveis destas mesmas citocinas após a ativação com TNF-α. Finalmente, para entendermos o impacto em termos de neurotoxicidade, realizámos co-culturas de microglia com neurónios do hipocampo, usando um sistema de microfluídica. Tanto a microglia estimulada com TNF-α como a microglia sobre expressando o miR-342, afetaram drasticamente a viabilidade dos neurónios. De notar que foram detetados níveis aumentados de nitritos nos sobrenadantes destas co-culturas. Globalmente, os nossos resultados identificaram o miR-342 como um importante mediador da ativação da microglia com TNF-α. Estes resultados estão compilados no artigo científico Brás *et al.*, *Cell Death and Disease* (2020).

De seguida, a fim de avaliar se o miR-342 estaria realmente desregulado e associado à depressão, utilizámos um modelo *in vivo* em que a exposição de ratos a eventos adversos durante a juventude leva a que estes desenvolvam e exibam comportamentos-tipo depressivos na fase adulta. Especificamente, exploramos como o stress peripubertal (PPS) combinado com diferentes perfis de resposta ao stress ao nível da produção de corticosterona (CSR), influenciam os comportamentos-tipo depressivos e a expressão de marcadores inflamatórios no cérebro, incluindo o miR-342. Descobrimos que ratos machos submetidos ao PPS e com produção exacerbada de corticosterona em resposta ao stress (H-CSR), exibiram comportamentos-tipo depressivos na fase adulta. Para além disso, nos animais submetidos ao PPS, foi detetado um aumento da expressão de TNF-α no hipocampo, que se correlacionou positivamente com respostas de *coping* passivo, em que os animais exibem falta empenho para lidar com exigências adversas externas. No mesmo sentido, o PPS causou também efeitos a longo-termo ao nível das células da microglia no hipocampo, particularmente nos ratos H-CSR, com aumento da expressão de IBA-1 e alterações morfológicas compatíveis com um maior grau de ativação. Por sua vez, nos ratos H-CSR que foram submetidos ao PPS foram detetados níveis aumentados de miR-342 no hipocampo. De notar que os níveis de expressão do miR-342 correlacionaram-se positivamente com os níveis de TNF-α, com o grau de ativação da microglia e com as respostas de *coping* passivo. Assim, estes resultados não só corroboraram, *in vivo*, as nossas descobertas anteriores de uma forte interação entre o miR-342, o TNF-α e a

ativação da microglia, como mostram também, pela primeira vez, uma desregulação da expressão do miR-342 no cérebro de ratos com comportamentos-tipo depressivos. Estes resultados foram compilados num artigo científico que está agora a ser revisto numa revista internacional com revisão por pares.

Por fim, realizámos um estudo clínico como prova de conceito para explorar o potencial de alguns miRNAs inflamatórios, particularmente o miR-342, como biomarcadores periféricos para o diagnóstico da depressão. Foram incluídos no estudo 40 indivíduos saudáveis e 32 doentes diagnosticados com depressão antes de iniciarem o tratamento. Os níveis de citocinas inflamatórias no plasma e os seus níveis de expressão nas células mononucleares do sangue periférico (PBMCs) foram avaliados. Para além disso, os níveis de expressão dos miR-145, miR-146a, miR-155 e miR-342 nas PBMCs foram igualmente medidos. Os resultados mostraram que a coorte de doentes depressivos apresentou um perfil pró-inflamatório no plasma, com níveis elevados de TNF-α e CCL2 em comparação com os indivíduos saudáveis. Nas PBMCs, detetamos um aumento e uma diminuição dos níveis de expressão de TNF-α e IL-6, respetivamente. Relativamente ao perfil de expressão dos miRNAs testados, encontrámos um aumento dos níveis do miR-342, enquanto que os níveis do miR-146a e do miR-155 estavam significativamente diminuídos nas PBMCs dos doentes depressivos. Digno de nota, os níveis de expressão de miR-342 correlacionaram-se positivamente com os níveis de TNF-α nas PBMCs. Por fim, quando testado o potencial destes miRNAs como biomarcadores para diagnóstico da depressão, o uso do miR-342, miR-146a, miR-155 em combinação foi o melhor classificador (AUC = 0,842, CI = 0,75-0,93), com potencial para constituir uma boa ferramenta de diagnóstico. Estes resultados foram compilados num manuscrito que está agora em fase final de preparação para submissão.

De uma forma geral, o trabalho desenvolvido nesta tese contribuiu para melhor compreendermos a interconexão entre a inflamação e a depressão. Para tal, explorámos com maior detalhe como os miRNAs regulam e aparecem desregulados em ambos os processos. Globalmente, os resultados aqui apresentados e discutidos, identificam o miR-342 como um potencial alvo diagnóstico e terapêutico, particularmente nos casos em que mediadores inflamatórios, como o TNF-α, estão sobre expressos. Descrevemos o mecanismo pelo qual o miR-342 contribui para a ativação da microglia via TNF-α e descobrimos que a sua expressão aparece desregulada no hipocampo de ratos com comportamentos-tipo depressivos e aumento de neuro-inflamação. Finalmente, encontrámos o miR-342 também aumentado nas PBMCs de doentes diagnosticados com depressão e, juntamente com outros dois miRNAs inflamatórios, o miR-146a e o miR-155, mostraram constituir um painel de biomarcadores com potencial para ser usado no diagnóstico da depressão. Estudos futuros devem investigar e validar estas descobertas

em coortes de pacientes mais robustas, a fim de definir o potencial destes e de outros miRNAs como biomarcadores específicos de diagnóstico e de resposta ao tratamento para a depressão.

List of abbreviations

5-HT	5-hydroxytriptamine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
Ago2	Argonaute 2
Amg	Amygdala
AMOs	Anti-miRNA antisense oligonucleotides
AU	Arbitrary units
AUC	Area under the curve
Aβ	Amyloid beta
BAG-1	Bcl2 associated athanogene 1
BBB	Blood brain barrier
BD	Bipolar disorder
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CA	Closed arm
CA1/3	Cornu ammonis 1/3
CCL	Chemokine (C-C motif) ligand
CD	Crohn’s disease
cDNA	Complementary DNA
CI	Confidence interval
CMS	Chronic mild stress
CNS	Central nervous system
CORT	Corticosterone
COX-2	Cyclooxygenase-2
Cq	Quantification cycle
CRP	C-reactive protein
CSR	Corticosterone-stress responsiveness
CTR	Control
CXCL	Chemokine (CXC motif) ligand
DALYs	Disability-adjusted life-years
DAVID	Database for Annotation, Visualization and Integrated Discovery
DG	Dentate gyrus
DGAV	Direcção Geral de Alimentação e Veterinária
DGCR8	DiGeorge syndrome chromosome region 8
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
FACS	Fluorescence-activated cell sorting
FADD	FAS-associated death domain protein
FBS	Fetal bovine serum
FC	Fold change
FITC	Fluorescein isothiocyanate
FST	Forced-swim test
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H-CSR	High-CORT stress responsive
HBSS	Hank's balanced salt solution
HPA	Hypothalamus-pituitary-adrenal
HPC	Hippocampus
IBA-1	Ionized calcium-binding adapter molecule 1
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IL	Interleukin
inflammiRs	Inflammatory miRNAs
IRAK1	IL-1 receptor-associated kinase 1
JAK	Janus kinase
JNK	C-jun N-terminal kinase
KLF13	Kruppel Like Factor 13
KYN	Kynurenine
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
lncRNAs	Long ncRNAs
LPS	Lipopolysaccharide
MAOIs	Monoamine oxidase inhibitors
MAPK	Mitogen activated protein (MAP) kinase
MDD	Major depressive disorder
MIF	Macrophage migration inhibitory factor
MIP-2	Macrophage inflammatory protein 2
MIQE	Minimum information for publication of quantitative real-time PCR experiments
miRNA	microRNA
mPFC	Medial prefrontal cortex
mRNA	Messenger RNA
MS	Multiple sclerosis
MSP	Maternal separation

MSR1	Macrophage scavenger receptor
MyD88	Myeloid differentiation primary response 88
N-CSR	Normative-CORT stress responsive
NAc	Nucleus accumbens
ncRNAs	Non-coding RNAs
NDS	Normal donkey serum
NeuN	Neuronal nuclei
NF-κB	Nuclear factor kappa B
NLRP3	NLR family pyrin domain containing 3
NMDA	N-methyl-D-aspartate
NO	Novel object
NOS2	Nitric oxide synthase 2
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Open arm
OF	Open field
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pCMV6	Entry, mammalian vector with C-terminal Myc
PD	Parkinson's disease
PE	Phycoerythrin
PFA	Paraformaldehyde
PPS	Peripubertal stress
pre-miRNAs	Precursor microRNAs
pri-miRNAs	Primary microRNAs
RA	Rheumatoid arthritis
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640 media
rRNAs	Ribosomal RNAs
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SacPT	Saccharin preference test
SCR	Mirna mimic negative control
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide electrophoresis gel
SERT	Serotonin transporter
SHIP1	Inositol polyphosphate-5-phosphatase
shRNAs	Short-hairpin RNAs

siRNAs	Small interfering RNAs
SIRT1	Silent information regulator 1
SMAD3	The mothers against decapentaplegic homolog 3
snoRNAs	Small nucleolar RNAs
SNR	Signal-to-noise ratio
SNRIs	Serotonin-norepinephrine reuptake inhibitors
snRNAs	Small nuclear RNAs
SocPT	Social preference test
SOCS1	Suppressor of cytokine signaling 1
SSRI	Selective serotonin reuptake inhibitors
STAT	Signal Transducer and Activator of Transcription
SZ	Schizophrenia
TCAs	Tricyclic antidepressants
TDO	Tryptophan 2,3 dioxygenase
TIRAP	Toll-interleukin-1 receptor domain-containing adaptor protein
TLR	Toll-like receptor
TMT	2,4,5-trimethylthiazole
TNF-α	Tumour necrosis factor
TRAF6	TNF receptor-associated factor 6
TRBP	Trans-activation-responsive RNA-binding protein
TREM	Triggering receptor expressed on myeloid cells 2
tRNAs	Transfer RNAs
UC	Ulcerative colitis
UTR	Untranslated region
WHO	World health organization
YLDs	Years lived with disability

I. General Introduction

1. Inflammation in the context of depression

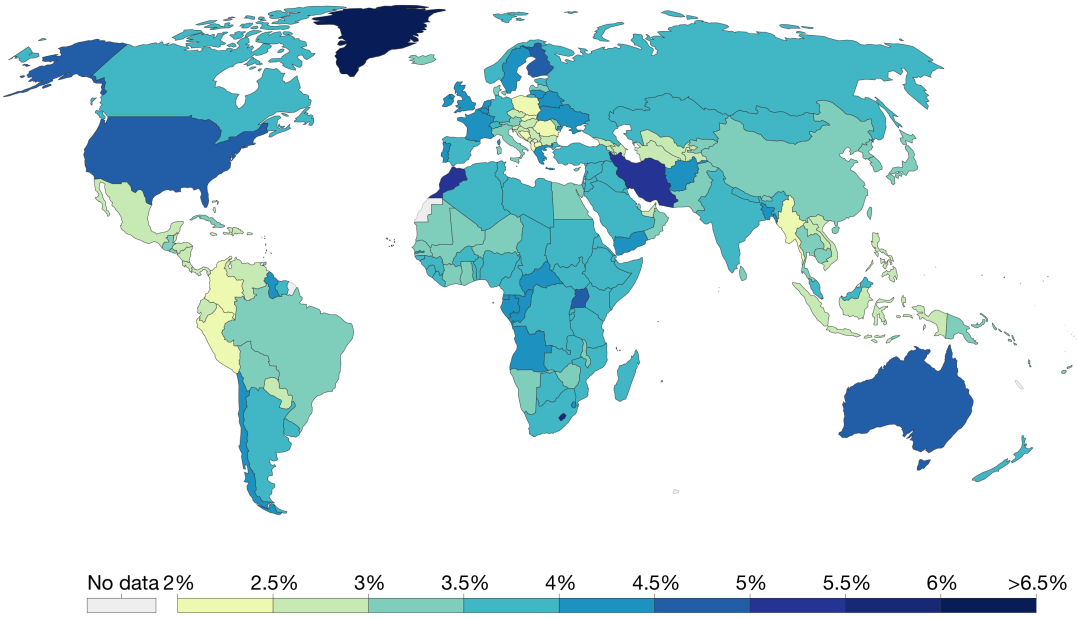
The content of this section was adapted from the book chapter published in the scope of this thesis:

Book Chapter published

João P. Brás, Sara Pinto, Maria I. Almeida, Joana Prata, Orlando von Doellinger, Rui Coelho, Mário A Barbosa, Susana G. Santos. Peripheral Biomarkers of Inflammation in Depression: Evidence from Animal Models and Clinical Studies. Methods Mol Biol. 2019;2011:467-492. doi: 10.1007/978-1-4939-9554-7_28.

1.1. Depression prevalence, classification, diagnosis and treatment

Depression is one of the most frequent mood disorders, affecting more than 264 million people of all ages worldwide (1). Published in 2018, The Global Burden of Diseases, Injuries, and Risk Factors Study 2017, that included a comprehensive assessment of incidence, prevalence, and years lived with disability (YLDs) for 354 causes in 195 countries and territories from 1990 to 2017, reported an increase in the number of people suffering from depressive disorders, with a prevalence that ranged between 2% and 6% around the world (2). According to the last numbers, Portugal with 4.42% ranks 3rd in Europe (Finland, 5.43% [1st]; Sweden, 4.49% [2nd]) and 11th worldwide (Greenland, 6.23% [1st]; United States, 4.84% [6th]) in the list of countries with higher prevalence of depressive disorders (2) (Figure 1).



Source: IHME, Global Burden of Disease

Figure 1. Depression prevalence worldwide in 2017. Greenland (6.23%) presents the higher and Colombia the lowest (2.20%) depression prevalence worldwide. In-continent variations reflect specific national realities, that are influenced by different factors. For instance, Central Europe (2.44%) depression prevalence average is considerably lower than in Western and Eastern Europe (3.92% and 3.80%, respectively). Source: Global Burden Disease 2017 (2).

Globally, depression prevalence tends to be higher in women (approximately 61% of all cases) and to increase with age, as older individuals have higher risk of depression relative to other groups (2). It is a leading cause of disability and a major contributor to the global burden of disease, associated with increased healthcare associated costs, poor health-related quality of life and increased mortality. In terms of YLDs, low back pain, headache disorders, and depressive disorders were the leading causes in 2017 for both sexes combined (2). Between 1990 and 2007, the number of all-age YLDs attributed to depressive disorders increased by 33.4%, becoming the third leading cause of all-age YLDs in 2007. From 2007 to 2017, further increases in the number of all-age YLDs attributable to depressive disorders were observed (14.3%). More recently, an updated analysis with detailed disease prevalence by age was released (3). Depressive disorders rank amongst the most prevalent causes of disability-adjusted life-years (DALYs) until the age of 49. Particularly, in adolescents aged 10–24 years, depressive disorders rank as the fourth top cause of DALYs, after road injuries, headache disorders and self-harm, with higher prevalence in females (3). With the burden of depression and other mental health conditions on the rise globally, the World Health Organization (WHO) passed a resolution in May 2013 calling for a comprehensive, coordinated response to mental disorders at the country level (4).

Depression differs from usual everyday life mood fluctuations in its intensity and duration and may result from the interaction of three major risk factors: personal or family history of depression; major life changes, trauma, or stress; and certain physical illnesses and medications. Stressful life events are the most frequent triggers of depression which, in turn, leads to more stress and dysfunction in a long-lasting negative cycle that worsens the affected person's situation. There are also interrelationships between depression and physical health. While depressive disorders may enhance the development and worsening of other medical conditions, such as coronary artery disease, atherosclerosis, diabetes and osteoporosis, the opposite can also happen (5-7). For over 75% of patients, major depression is a recurrent disease, 50% of patients with no maintenance therapy present an exacerbation within six months of the first episode, and 15% of untreated patients succumb to suicide (8). Almost 800 000 people died due to suicide in 2015, being the second leading cause of death in 15-29-year-olds. Men are twice as likely as women to die as a result of suicide (9).

Depressive disorders comprise different clinical entities: major depressive disorder/episode, mild persistent depressive disorder (dysthymia), premenstrual dysphoric disorder, substance/medication-induced depressive disorder, depressive disorder due to another medical condition, other specified depressive disorder, and unspecified depressive disorder. Overall, they are characterized by a general feeling of sadness, lack of pleasure or interest in activities, changes in weight and appetite, sleep disturbances, difficulty in concentrating, and feelings of worthlessness and hopelessness, with or without suicidal ideation (10). Traditional diagnostic tools include physician-administered or patient self-administered clinical interviews that, based on the number of reported symptoms and threshold scores, are often used to classify individuals as healthy or depressed (11). However, these methods have raised major concerns, considering that specific depressive symptoms like those mentioned above, are distinct phenomena that differ from each other in important dimensions such as underlying biology, impact on impairment, and risk factors (12). The generalized use of rating scales scores to estimate depression has therefore limited the identification of new biomarkers and more efficacious antidepressants. Current treatment guidelines for depression are based on psychotherapeutic interventions and antidepressant drugs administration. Psychotherapeutic interventions include behavioural activation, cognitive behavioural therapy, interpersonal psychotherapy and supportive therapy (13). Different psychological treatment formats may include individual and/or group face-to-face psychological treatments delivered by professionals and supervised lay therapists. However, these approaches are only particularly effective in cases of mild depression or as adjuvant therapies (14). In cases of moderate to severe depression, antidepressants, particularly those targeting neurotransmitters that are altered in the central

nervous system, such as selective serotonin reuptake inhibitors (SSRI), serotonin-norepinephrine reuptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs), are the first treatment line of choice (15). Despite the efficacy of such interventions, one third of patients does not respond to the first treatment scheme, and at least one third of those patients is resistant or intolerant to those therapies (16). There are several barriers to effective care that include a lack of resources, lack of trained health-care providers and social stigma associated with mental disorders. However, the most important hurdle still preventing more effective treatments is inaccurate diagnosis. Due to the multifactorial aetiology of this disorder, there are pathological mechanisms underlying depression that need to be further clarified. This will provide new diagnostic tools with regard to the biological particulars of each patient, allowing a move away from narrative and observation-based diagnosis, thereby increasing treatment efficacy rates.

1.2. The inflammatory theory of depression

Depression pathophysiology is complex and multifactorial. There is a significant genetic influence, as well as the unbalance of several endogenous systems, namely the hypothalamus-pituitary-adrenal axis and the immune system, along with external factors (17). Depressive disorders have been recurrently associated with an increase in systemic levels of biomarkers of inflammation (5), with studies reporting that most treatment resistant patients present a hyper-activation of the immune system (18), namely an increase of pro-inflammatory biomarkers (19-22). It has been reported that severe stressors, both psychological and physical, may trigger depression-like and pro-inflammatory states, which improve with antidepressant treatment (5, 23). Similarities have been highlighted between depression and “sickness behaviour”: when individuals suffer from infectious or inflammatory diseases, there is a change in their behaviour, with anorexia, anhedonia, isolation and insomnia; they also feel depressed and irritable, with difficulties in maintaining concentration (24, 25). This clinical scenario occurs in response to an increase in pro-inflammatory cytokines, such as interleukin-1α and β (IL-1α and IL-1β), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which in the sickness context enables the conservation of energy, redirecting it towards the resolution of the challenge faced (26). However, in depression it presents a chronic maladaptive behaviour (27).

The approach to depression as a maladaptive extension of the “sickness behaviour” was first made by Hart BL. (25) and was later explored by RS Smith in his article “The macrophage theory of depression” (1991) (28). He stated that the activation of the immune system was a cornerstone in the pathophysiology of depression, suggesting that the secretion of macrophage cytokines, namely IL-1β and TNF-α, promoted the development

of some cases of major depressive disorder (28). Subsequent studies highlighted that depressed patients present higher levels of inflammatory biomarkers in peripheral blood, including cytokines, C-reactive protein and other acute phase molecules (29-31). This was further complemented with the knowledge on inflammatory monocytosis during chronic stress states. Monocytes may infiltrate the central nervous system (CNS) and differentiate into macrophages, where tissue resident macrophages and dendritic cells, can also be found (32). The migration of such cells is potentiated in situations of chronic physical or psychological stress, likely due to disruption of the brain blood barrier, and may interfere in neuronal networks essential for the development of depression or anxiety disorders (33, 34). In fact, a study performed in post-mortem brains showed that individuals previously diagnosed with depressive disorder presented an increased number of monocytes in the CNS, suggesting a higher cell recruitment rate from peripheral blood (35).

In clinical context, the therapeutic administration of some cytokines also induces depressive behaviour. Almost 50% of previously non-depressed cancer patients treated with recombinant IL-2 present major depressive disorder (36, 37). The observation of the clinical course of these patients highlighted that in the beginning all developed a clinical scenario similar to the “sickness behaviour”, with neurovegetative and somatic symptoms of depression (38, 39). Moreover, in patients with cancer, hepatitis C virus, multiple sclerosis and other autoimmune disorders, which received prolonged interferon (IFN)-based treatments, a form of “the IFN-induced depression” could be observed (40, 41). About one fourth to one third of patients with hepatitis C treated with IFN-α develop depressive symptoms (42). This type of depression shares many symptoms and features with major depression (43, 44), and upon diagnosis, IFN-induced depression responds to conventional treatments that are effective for idiopathic major depression (45-48).

1.3. Inflammatory molecular mechanisms of depression

There are several lines of evidence, regarding the effect of persistently high cytokine levels in the anatomy and physiology of the CNS. First, pro-inflammatory cytokines such as IFN-γ, IFN-α, IL-1β and TNF-α can up-regulate the expression of serotonin transporter (SERT) in the brain, increasing 5-hydroxitriptamine reuptake, with a reduction in extracellular concentrations (49, 50). In a mouse model, IL-1β and TNF-α enhanced serotonin uptake in midbrain and striatal synaptosomes, through the activation of the p38 mitogen activated protein (MAP) kinase, while IFN-α decreased the expression of serotonin receptor 1A in various non-neuronal cell lines (50). Pro-inflammatory cytokines IL-6 and IL-1β also exert a direct action on astrocytes, microglia and neurons throughout the CNS (51, 52). Microglia cells are the CNS parenchymal macrophages and comprise about 10% of all

brain cells (26, 53). They are maintained in a quiescent state, though constantly and dynamically supervising the surrounding environment (54), promoting synaptic pruning (55, 56) and detecting neuronal damage (57). This microglial response is intimately related to the activity of the peripheral immune system. Systemically produced cytokines may promote the activation of microglial cytokine production, alongside with an alteration of astrocyte transcriptome profile (58, 59), through either peripheral or central stimuli. This creates a cerebral translation of the peripheral immune deregulation (26). When exposed to inflammatory stimuli or tissue damage, microglia cells produce different types of mediators, namely chemotactic factors, leading to the recruitment of circulating monocytes (60, 61) and to the activation of other neuronal and non-neuronal brain cells. Microglia loss and peripheral monocyte infiltration into the CNS have been documented in animal models of depression and anxiety, as enhancing the local inflammatory response (62, 63) (Figure 2).

Both intense activation or reduction/suppression of microglia number or activity in certain brain regions may be related to the development of depressive disorders (53). Immune challenges performed in humans (e.g. with the administration of lipopolysaccharide (LPS) or Salmonella typhi that are known to trigger an intense microglial activation) lead to the development of depressive symptoms, which are directly proportional to the increase of plasma levels of inflammatory cytokines (20, 64, 65). Furthermore, microglia from stressed rodents released higher levels of IL-1β and IL-6 after *in vitro* stimulation with LPS (66, 67). This pro-inflammatory microglial activation may be further potentiated by glucocorticoids, when administrated previously to the immune challenge, since it increases the release of TNF-α, IL-1β and IL-6 from the brain resident macrophages (68).

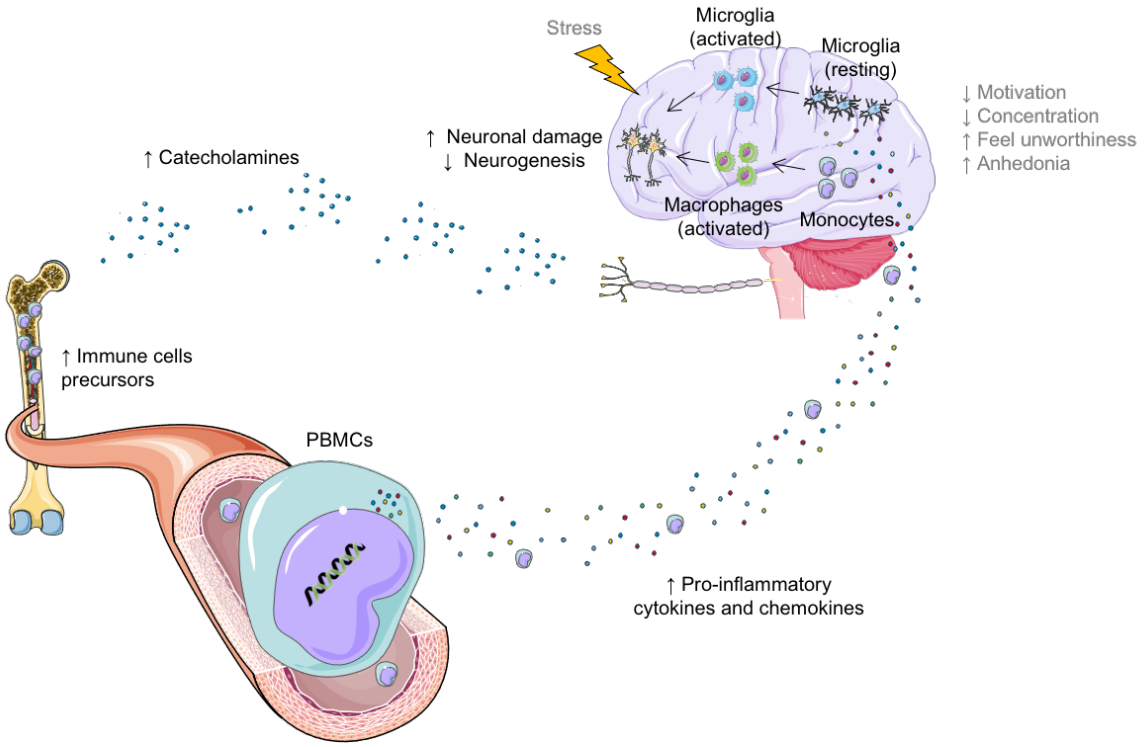


Figure 2 - Transmitting stress-induced inflammatory signals to the brain. In response to physiological and psychosocial stressors, sympathetic nervous system fibres release catecholamines (such as norepinephrine) that stimulate myeloid cells formation and modulate immune response gene transcription through stimulation of β-adrenergic receptors. This adrenergic signaling up-regulates transcription of proinflammatory cytokines, such as IL-1β, TNF-α, and IL6, leading to increases in systemic inflammatory activity. These cytokines can access the brain through humoral and neural routes where they lead to the activation of microglia to a pro-inflammatory phenotype. Activated microglia release neurotoxic and inflammatory mediators, such as CCL2 that in turn attracts activated myeloid cells to the brain. Once in the brain, activated microglia and macrophages can perpetuate central inflammatory responses that promote neuronal damage, decrease neurogenesis and impact normal neurocircuits. These neuro-immunological alterations ultimately lead to the development behavioural deficits characteristics of depression (adapted from (69)).

Besides microglia, astrocytes are involved in the pathophysiological mechanisms of depressive disorders. These cells are decreased in brain regions responsible for emotional control, as shown by analysis of brain samples retrieved from post-mortem depressed humans or from rodents previously subjected to stress protocols (70, 71). Some hypothesize that this reduction may not reflect a real decrease in astrocyte number, but a decrease in astrocytic volume and branching induced by chronic stress (72). The morphological change of astrocytes may compromise the effective role of the brain blood barrier, with increased permeability to circulating components (73).

The inflammatory response is also related with the disturbance of the stress system in depressive disorders. It has been shown that different components of the immune response, particularly pro-inflammatory cytokines, may potentiate the activity of the hypothalamus-

pituitary-adrenal (HPA) axis. In chronic inflammatory conditions, a pro-inflammatory environment might induce glucocorticoid resistance in immune cells, as well as in some of their targets, mostly through the MAP kinases c-jun N-terminal kinase (JNK) and p38 pathways (74). In the CNS, inflammatory cytokines increase the expression of the β isoform of the glucocorticoid receptor, increasing the binding of cortisol to this inactive isoform, thus interfering with the negative feedback response mediated by the α isoform of the glucocorticoid receptor (75, 76). Concerning the immune system, both innate and adaptive immune cells express both adrenergic and glucocorticoid receptors (76, 77), which act as fine regulators of the immune response, preventing a generalized spread of the inflammatory response through the so called “inflammatory reflex” (78). More precisely, glucocorticoids prevent the activity of nuclear factor-kappa B (NF-κB), a key transcription factor for inflammatory response, thus inhibiting the production of pro-inflammatory cytokines. In depressive disorders, despite the increase in cortisol levels, there is a diminished response of immune cells and microglia to corticoids due to altered intracellular pathways (79, 80). Thus, this glucocorticoid resistance in immune cells, particularly from the innate arm (81, 82), translates into a decrease of the inhibitory effect of these hormones over cytokines production, triggering reactive granulopoiesis (83), with an increase in neutrophils and monocytes number, and potentiating the overall inflammatory response. In animal models of stress-induced depression, stress induces a desensitization of glucocorticoid receptors present on splenic monocytes (reviewed in (84)), along with an increase in circulating cytokines and the presence of anxiety-like behaviour. This deregulated glucocorticoid response and regulation triggers a vicious circle, with inflammation and stress potentiating each other (26). Furthermore, most studies reporting increased peripheral cortisol and inflammatory response also evidenced glucocorticoid resistance and this was observed in 85% of depressed patients with increased levels of pro-inflammatory cytokines, even in the absence of peripheral hypercortisolaemia (85).

Another mechanism that links inflammation and depression is the disruption of the tryptophan metabolism through the kynurenine (KYN) pathway. Tryptophan is essential for the synthesis of serotonin. This amino acid is actively transported from the plasma to the CNS. Both stress and immunotherapy lead to the activation of tryptophan 2,3 dioxygenase (TDO) and indoleamine 2,3 dioxygenase (IDO), which are rate limiting enzymes to the metabolism of tryptophan through the KYN pathway (50, 86). These enzymatic reactions are regulated by cytokines, steroids, and growth factors, and occur both at the periphery and in the CNS, particularly in astrocytes and microglia (86). Specifically, pro-inflammatory cytokines change monoamine metabolism by increasing expression and function of the reuptake transporters, which reduces the amount of available monoamines (serotonin, dopamine and noradrenaline) (87). This is particularly critical since the monoamine

transporters are the antidepressant target for monoamine reuptake inhibition (88). The KYN metabolic pathway leads to the production of metabolites such as: KYN, kynurenic acid, 3-hydroxykynurenine and quinolinic acid, by activated microglia and infiltrating monocytes and macrophages (89, 90). Like tryptophan, KYN, and 3-hydroxykynurenine may cross the brain-blood barrier through specialized transport mechanisms and are further metabolized, being responsible for most of KYN metabolites in the CNS (91, 92). Here, 3-hydroxykynurenine may also react with the enzyme xanthine oxidase, leading to the production of reactive oxygen species, with subsequent oxidative damage (93). Additionally, quinolinic acid acts as an agonist of the glutamatergic N-methyl-D-aspartate (NMDA) receptor and, in high concentrations, may induce excitotoxicity due to over-activation of these receptors, leading to increased calcium ions influx and subsequent neuronal damage (94). IDO is present in every organ system, particularly in macrophages and dendritic cells, and can be directly activated by several cytokines, including IFN-γ, IL-1β and TNF-α (26, 95, 96). During chronic inflammation, there is an increased peripheral degradation of tryptophan, with higher levels of circulating kynurenine, 3-hydroxykynurenine and quinolinic acid, which interfere with the peripheral immune system and may increase the KYN metabolites level in the CNS (97-99). Furthermore, inflammatory states increase the permeability of the brain-blood barrier, allowing the entrance of cytokines and macrophages in the CNS, both capable of increasing tryptophan metabolism through KYN pathway (100). As a result, the deleterious effects in the CNS of 3-hydroxykynurenine and quinolinic acid, as well as the decreased level of tryptophan available for the synthesis of 5-hydroxytriptamine (5-HT), causes an unbalanced scenario in depressive disorder. Depressed patients present decreased peripheral levels of tryptophan, which correlates with decreased central levels of 5-HT (101-103). It has been shown that the reduction in plasma levels of tryptophan correlated with the patients' depression scores after three weeks of treatment (26). Furthermore, quinolinic acid-NMDA receptor induced excitotoxicity is particularly important in the hippocampus, being related to the cognitive and memory alterations in depressed patients. Moreover, it has been shown that neural activity in brain areas affected by the 5-HT system, such as the dorsal raphe, habenula, septal region, amygdala, and orbitofrontal cortex, is intrinsically related to plasma tryptophan levels and with depressed mood scores (5).

Furthermore, both pro-inflammatory cytokines and quinolinic acid directly affect glutamate metabolism, by decreasing astrocyte-mediated glutamate reuptake and stimulating its release (104). Excessive glutamate leads to decreased synaptic plasticity and production of brain-derived neurotrophic factor (BDNF), a critical molecule involved in neurogenesis (105). Equally important in the pathogenesis of depression, is the impact that inflammation and specifically, pro-inflammatory cytokines, have in the neuroendocrine

system. Markedly, pro-inflammatory cytokines contribute to HPA axis hyperactivity, by suppressing glucocorticoid receptor and thereby preventing negative feedback mechanism (106).

1.4. Depression in chronic inflammatory diseases

The prevalence of depression is increased in several auto-immune/auto-inflammatory diseases, like psoriasis, inflammatory bowel disease (IBD), or rheumatoid arthritis (RA).

Psoriasis is a common chronic immune-mediated inflammatory disease, characterized by increased proliferation of keratinocytes and inflammatory leukocyte cell infiltration into the epidermis and the dermis (107, 108). The prevalence of depression and/or anxiety disorder in psoriasis patients ranges from 30% to 58% (109, 110). High stress levels are risk factors to the onset of psoriasis (111) and disease flares (110, 112). Moreover, effective psychiatric treatment leads to a decrease in the number and frequency of psoriasis symptoms as well as reduced need for ultraviolet radiation therapy (113-116). Psoriasis treatment also appears to promote an improvement in depressive symptoms (117).

IBD comprehends different chronic inflammatory conditions, associated with a disruption of the intestinal mucosa homeostasis driven by the cells of the adaptive immune system, in response to self-antigens. Crohn's disease (CD) and ulcerative colitis (UC) are the most prevalent and better-known conditions within IBD (118). Studies have suggested that a dysfunctional brain-gut interaction could be involved in the pathogenesis of IBD (119, 120). On the one hand, stress exposure (e.g. stressful events and perceived stress) potentiates relapse risk, while active symptomatic disease may exacerbate or even incite stress. Induction of depression in an animal model of colitis was associated with reactivation of inflammation in those with previously established quiescent chronic inflammation. This effect was partially mediated by an increase in pro-inflammatory cytokine secretion by macrophages and prevented by tricyclic antidepressants administration (121). IBD patients presented fewer relapses and required less steroid treatment in the year after starting antidepressant therapy, possibly due to an inflammation-related benefit from the use of antidepressants (122). In the case of UC, patients are more likely to suffer from depressive disorders before the onset of UC (123). At a molecular level, there is a positive correlation between anxiety and depression and the induction of the antiapoptotic protein HSP70 in polymorphonuclear cells, which is a heat shock protein known to promote inflammation in UC patients (124). Regarding the relation between CD and depressive disorders, it has been shown that levels of depressive symptoms were positively associated variations in Crohn's Disease Activity Index (125) and the total number of relapses (126).

RA is an autoimmune disease characterized by joint swelling; osteoarticular deformity; production of the autoantibodies rheumatoid factor and anti–citrullinated protein antibody (ACPA); and systemic co-morbidities, namely cardiovascular, pulmonary, psychological and skeletal (127). Depression is more prevalent in RA patients than in the general population (128) and it has been associated with increased morbidity and mortality in these patients (129-131). Besides possible common endogenous etiopathogenic pathways, potential causes for the development of depression in RA patients include age, severity of the disease, pain, and work disability (132). On the other hand, the presence of depressive disorder/depressive symptoms enhances the subjective negative perception of disease consequences (132), and a recent retrospective cohort study suggests that depression increases the risk of developing RA and that anti-depressant medication may mitigate such risk (133).

Multiple Sclerosis (MS) is a chronic autoimmune disease of the central nervous system, characterized by inflammation, demyelination, and neurodegeneration in the brain and spinal cord (134). Depression presents in over 50% of MS patients over a life-time (135), being one of the most debilitating co-morbidities (136-140). Besides the disease related adverse life events associated, which increase the risk for the development of depressive disorders, there appear to be some common pathophysiological processes, namely the dysregulation of the inflammatory and immune response. Similar to depressive disorders, multiple sclerosis also presents cytokine imbalance as well as alterations of B and T cell phenotype and function.

1.5. Clinical evidence: anti-inflammatory drugs for depression treatment

Several clinical trials have provided evidence of the efficacy of different anti-inflammatory drugs in depressive symptoms/disorder, most of them correlating with the basal inflammatory profile of the patients. Thus, infliximab, a monoclonal antibody against TNF-α, was shown to be effective for improving mood in depressed patients with increased basal inflammation levels, translated by C-reactive protein circulating levels (141). Such improvement has also been observed in individuals with depression and psoriasis in co-morbidity treated with ustekinumab, an antibody against interleukin 12/23 (142). Tocilizumab, a humanized antibody to the IL-6 receptor approved for the treatment of arthritis and Castleman's disease, is being considered as a therapeutic option for unipolar and bipolar depression (143). The role of non-steroidal anti-inflammatory drugs (NSAIDs) on depression is also being assessed. Some studies have shown that treatment with these compounds, particularly the cyclooxygenase-2 (COX-2) inhibitor celecoxib, decreases the

symptoms of depression (144, 145). However, these results are still controversial, with the use of some substances being even detrimental in depressed patients (e.g. ibuprofen interferes with the bioavailability of selective serotonin reuptake inhibitors) (146). A new clinical trial protocol was recently registered (ACTRN12617000527369), aiming to evaluate the effect of combining a serotonergic anti-depressant (vortioxetine) with NSAID anti-inflammatory (celecoxib) therapy in c-reactive protein (CRP) high and low depression patients (147). However, the study has faced participant recruitment difficulties and, as a mean to have enough eligible patients, the authors recently published a report on the clinical switching strategies of various antidepressants to vortioxetine (148). Of 122 study participants randomized to receive either vortioxetine plus celecoxib or vortioxetine plus placebo, 82 were taking a different antidepressant medication prior to commencing the study (148). A cross-titration method was used to change study participants from their current antidepressant medication to vortioxetine, a method that was generally well-tolerated (148). Nonetheless, results on the contribution of celecoxib to augment vortioxetine therapeutical effects are yet to be released.

Other line of research focuses on the immunomodulatory properties of antidepressant drugs. Some studies concluded that antidepressants had an anti-inflammatory action (149), while others reported either no action (150) or even a potentiation of the inflammatory response (151). Interest has grown regarding the drug ketamine. It presents as a new type of antidepressant treatment effective on treatment-refractory depression (152), which promotes a decrease in several pro-inflammatory cytokines, namely IL-6 and TNF-α, partly through the Toll-like receptor (TLR) 4 family (153).

1.6. Inflammatory biomarkers for the diagnosis and monitoring of depression

Over the last decades, the link between inflammation and depression has been explored in the search for quantifiable biological biomarkers of disease, that can be detectable peripherally, in a minimally invasive way, and also to understand the pathophysiological mechanisms responsible for treatment resistance. Consequently, several inflammatory mediators, including, cytokines (e.g. IL-1β, IL-6 and TNF-α), chemokines (e.g. chemokine (C-C motif) ligand (CCL) 2 and chemokine (CXC motif) ligand (CXCL) 8, also known as MCP-1 and IL-8, respectively) and acute phase reactant proteins (e.g. CRP) have been highlighted as potential biomarkers for the diagnosis and monitoring of inflammation-based depression (154, 155). Also, inflammation has been associated with a decreased response to conventional antidepressants (21, 156, 157). The relevance of these peripheral inflammatory biomarkers in depression pathophysiology, as well as the evidences resulting

from animal studies, will be discussed in detail in this section and are summarized in Table 1.

1.6.1. Peripheral inflammatory biomarkers: clinical studies

a) Cytokines

Cytokines play a major role in inflammatory alterations. Increasing evidences indicate that cytokines are involved in the development of depression (69). Whether inflammation and cytokine levels are cause and/or consequence of a depressive disorder, remains to be fully established. However, as discussed above, chronic inflammation leads to the release of increased levels of cytokines into the peripheral circulation, which can ultimately access the brain and have a direct impact on neurotransmitter systems, neuroendocrine functions, and synaptic plasticity (154, 158).

Reports based on clinical evidences have been showing that patients with major depression exhibit increased levels of peripheral blood inflammatory cytokines, specifically IL-1β, IL-6, TNF-α and IFN (type I and II) (159-161). A systematic review of articles published between 1967 and 2008 to assess the magnitude and direction of associations of depression with IL-1β and IL-6 in clinically depressed patients revealed a positive association, even after adjusting for body mass index and other confounding factors (162). Interestingly, Dowlati *et al.* also reported a significant upregulation of IL-6 along with TNF-α in patients diagnosed with depression when compared to control subjects (163). In fact, evaluation of cytokine levels has not only been performed to help identifying depressive symptoms but also to monitor clinical response to treatment. Strawbridge *et al.* performed a meta-analysis comprising 35 studies that investigate inflammation before and after treatment in depressed patients together with a measure of clinical response (164). The authors found that levels of IL-6 decreased with antidepressant treatment, whereas persistently elevated TNF-α levels were associated with treatment resistance (164). Similar conclusions were taken by Hannestad and colleagues which found that while pharmacological antidepressant treatment was efficient on reducing depressive symptoms and serum levels of IL-1β and IL-6, it did not reduce serum levels of TNF-α (165).

It is widely described that cytokines can access the CNS through a variety of routes and affect monoaminergic systems involved in the pathology of depression (154, 166). Thus, their peripheral levels may be correlated with disease state and might be useful in monitoring treatment efficiency.

b) Chemokines

Chemotactic cytokines (chemokines) act as chemoattractants to guide cell migration. Considering their well-known role in immune cells recruitment (167, 168), brain-specific functions of these molecules have been lately studied (169-172). For instance, CCL2 and CCL3 have been implicated in neurotransmitter alterations (173-175); CXCL8 and CXCL10 in hypothalamic–pituitary–adrenal axis functions (176); CXCL12 and its receptor, CXC motif chemokine receptor type 4 (CXCR4) have been repeatedly identified as crucial players in neurogenesis (177); and finally chemokine (CX3C motif) ligand (CX3CL) 1, CCL2 and CCL21 have been highlighted to participate in microglia to neurons communication (178). Following these evidences, the hypothesis that chemokines may be important players on the crosstalk between peripheral immune system and central nervous system, mediating depressive behaviours, has emerged. Thus, peripheral blood levels of several chemokines have been quantified and pointed as potential biomarkers for depression diagnosis.

Studies evaluating peripheral chemokine alterations in depression, identified CCL2 and CXCL8 as the most consistent chemokines to be associated with depression (170, 179-182). Köhler *et al.* analysed 82 studies comprising 3212 participants with major depressive disorder and 2798 healthy controls (180). Among other dysregulated cytokines, peripheral levels of CCL2 were significantly elevated in patients with depression compared with healthy subjects (180). In its turn, Leighton *et al*. found that blood CCL2 (meta-analysis of 21 studies) and CXCL8 (40 studies) measurements were significantly higher in depressed subjects compared with controls (183). Importantly, this was further confirmed when applying sensitivity analyses to studies where only physically healthy patients participated, excluding the possibility of the association to be related with any other physical comorbidity (any comorbidity with an established inflammatory component) (183). Intriguingly, comparisons of CXCL8 levels in plasma and serum revealed a significant difference in plasma, but not serum (183), which is in agreement with previous findings (181). Other evidences also suggest that CCL3, CCL11, CXCL4 and CXCL7 are increased in depression (183-185), whereas CCL4 is negatively associated to the disease (181, 183). However, due to the small sample size, these findings should be further investigated. Considering the relationship between antidepressant treatment response and chemokines, it is noteworthy that regarding CXCL8, any study so far found a significant correlation (18, 186, 187). In contrast, CCL2 was recently detected as being elevated in treatment-resistant depressive subjects (188).

c) C-reactive protein (CRP)

CRP is an acute-phase protein that is produced in the liver by hepatocytes, upon stimulation by the cytokines IL-1β, TNF-α and IL-6, during an inflammatory response (189). It is commonly used as a biomarker of inflammation to predict and monitor several diseases (190). There are two tests for measuring CRP. The high-sensitivity C-reactive protein (hs-CRP) measures inflammation in blood vessels and is used to establish heart disease risk. hs-CRP levels of <1, 1 to 3, and >3 mg/L correspond to low-, moderate-, and high-risk groups for future cardiovascular events (191, 192). The other test can show a non-specific elevation of CRP that occurs with general inflammatory changes in the body. People having inflammatory disorders such as certain arthritic, autoimmune or inflammatory conditions, tend to have elevated CRP levels. In these cases, physicians evaluate inflammation with a test that measures levels in excess of 10 mg/L (192).

Recently, CRP levels have been also applied in the diagnosis of inflammation-associated depression. Although some results are conflicting, elevated CRP levels have been associated with psychological distress and depression (193, 194). Several studies with 5000 to 10000 participants found an increase of CRP levels in patients diagnosed with depression; however, in some cases, this association vanished after adjusting the values for confounding factors, such as chronic illness and body mass index (BMI) (195-197). To overcome the limitations of previous studies, Wium-Andersen *et al.* tested the same hypothesis by measuring CRP levels in a larger cohort of patients composed by 73 131 individuals from two independent general population studies, stratified into 4 clinically relevant categories and symptoms of psychological distress and depression (193). To reduce the influence from confounding factors, analyses were adjusted for a large number of parameters (age, sex, alcohol intake, smoking, BMI, register-based chronic disease, and others) (193). Using a cross-sectional analysis, these authors found that CRP levels were associated with increased risk for psychological distress and depression in general population. For self-reported use of antidepressants, the odds ratio was 1.38 for CRP levels of 1.01-3.00 mg/L, 2.02 for 3.01-10.00 mg/L, and 2.70 for greater than 10.00 mg/L compared with 0.01-1.00 mg/L. For prescription of antidepressants, the corresponding odds ratios were 1.08, 1.47, and 1.77, respectively; for hospitalization with depression, 1.30, 1.84, and 2.27, respectively (193). In prospective analysis, increased CRP levels were also associated with increasing risk for hospitalization with depression (193). A recent study also used CRP levels to predict response to antidepressant lurasidone and found that elevated CRP prior to treatment were associated with enhanced clinical response in patients with bipolar I depression (198).

These data corroborate the findings that blood levels of IL-1β, TNF-α and IL-6, inductors of CRP synthesis, are increased in depression. Moreover, since CRP has longer plasma half-life than cytokines (190), and directly reflects the intensity of the pathological processes that stimulated its production, CRP has been increasingly considered as a biomarker for the diagnosis and treatment response monitoring of inflammation-associated depression (141, 193, 199).

1.6.2. Peripheral inflammatory biomarkers: evidence from animal models

Given the complexity of symptoms, and incomplete comprehension about the molecular mechanisms of depression, the development of adequate animal models is challenging. Nevertheless, several pathophysiological features of depression, including risk factors such as stress exposure, neurotransmitters and hormonal dysregulation, as well as characteristic symptoms such as sociability, anhedonia and helplessness behaviours, have been reproduced in animal models (200). For instance, prenatal and early-life stress models have been used to study how early experiences and environmental factors impact developmental processes implicated in depression and how it correlates with inflammation dysregulation (201). Prenatal stress was reported to induce a reduction of BDNF expression in the prefrontal cortex and striatum of adult rats (202). Interestingly, in adulthood, animals displayed altered regulation of BDNF expression in these structures, indicating that adverse life events during gestation may interfere with BDNF expression and function in a region-specific manner (202). Moreover, prenatal stress increased hippocampal IL-1β mRNA levels and the number of reactive microglial cells in adult brains of C57BL/6 mice (203). In fact, it was also implicated in increased mRNA levels of IL-6, TNF-α, and IL-10 in the hippocampus of mice with peripheral inflammation induced by systemic administration of LPS. In turn, the early life period is known to be a critical time window in brain development that is sensitive to the deleterious effects of adverse experiences. An enhanced risk of developing depression in adulthood following stress exposure in early life has been reported in several studies (204, 205). Early postnatal stress models involving maternal separation (MSP), have been shown to induce long-term depressive-like behaviours (206). However, findings reporting the long-term impact of MSP on peripheral and brain inflammation have been inconsistent. While MSP has been shown to have an immediate impact on inflammation by increasing peripheral inflammatory markers and neuroinflammation in key brain regions implicated in depression (207), reports on the long-term effect are contradictory (207, 208). During the peripubertal period, comprising childhood and adolescence, stress (peripubertal stress, PPS) was also shown to enhance anxiety-related

behaviours and increased passive coping responses in adult rats (209, 210). However, the long-term effect of PPS in brain and peripheral inflammatory status is still unknown.

Furthermore, the development of depressive symptoms during adulthood has been studied in rodents using different approaches, including social isolation, learned helplessness, chronic mild stress and social defeat. Animals subjected to prolonged social isolation exhibited depression-like behaviours, avoiding the central zone in the open field test and being immobile longer in the forced swim test (FST) (211). In turn, the learned helplessness model induced behavioural deficits in response to uncontrollable stressors, resulting in a deficit in escaping from an aversive situation (212, 213). Following similar methodology, the chronic mild stress (CMS) approach has been extensively used and improved over the years (214). This model involves rodent exposure to mild unpredictable stressors (e.g.: intermittent illumination, food and water deprivation, variable housing, cold stress and forced swimming), randomly ordered, over several weeks (215). Stressed animals exhibited long-term depressive symptoms resulting from neuroendocrine and neuroimmune alterations and reflected in increased levels of circulating pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α (216). Finally, the social defeat stress model consists in repeated introductions in the cage of a larger and aggressive animal, which results in the development of a clear depressive-like syndrome, characterized by enduring deficits in social interactions (217). Repeated exposure to social defeat stress has been also associated with increased accumulation of neutrophils and monocytes in circulation, which could be explained by the observed increase of inflammatory cytokines IL-6 and TNF-α, and CCL2 (218) .

These evidences showing that inflammation is intimately correlated with depressive symptoms corroborate clinical observations. However, researchers have continued to improve rodent models to better study the hypothesized bidirectional association between immune activation and depressive symptoms, and ultimately understand the pathophysiological features that lead to treatment resistance. Therefore, over the last years, the theory of inflammation-associated depression has been extensively studied by combining depression models and administration of inflammatory agents (219). Growing literature demonstrates that local and systemic inflammation also leads to increased sickness, anhedonia, and anxiety-like behaviours in rodents (219, 220). Peripheral administration of LPS was reported to activate IDO culminating in a distinct depressive-like behavioural syndrome, characterized by increased duration of immobility in mice. LPS increased expression of brain TNFα, IL-1β, and IFN-γ mRNAs, all of which were inhibited by pre-treatment with minocycline (221). A recent study has shown that T lymphocytes and the brain IL-10 signaling were necessary to resolve depression on LPS treated animals (222). In turn, Goshen *et al.* explored the involvement of IL-1 in chronic stress-induced

depression (223). Mice subjected to CMS for 5 weeks exhibited depressive-like symptoms, with increased IL-1β levels in the hippocampus. Interestingly, exogenous subcutaneous administration of IL-1β mimicked the effects of CMS on both behavioural depression and neurogenesis (223), which was lately attributed to actions of the transcription factor NF-κB (nuclear factor-kB) (224). Also, central administration of IL-6 produced depressive-like phenotypes in mice which could not be reverted with fluoxetine (225). Sickness and depressive behaviours triggered by inflammation have been abrogated and attenuated by anti-inflammatory agents (221, 226, 227). For example, NSAIDs, including diclofenac and celecoxib, have been shown to modulate and reverse IFN-alpha-induced depressive symptoms (228, 229). In addition, Karson and colleagues found that chronic administration of a TNF-α inhibitor reduced depression and anxiety-like behaviour in CMS model of depression in rats (230).

Thus, rodent models have brought to knowledge new evidences about the bidirectional association between inflammation and depression, which should be further investigated and maturated in order to identify new molecular targets with potential to be translated into the clinics in the form of new, more effective diagnostic and treatment methods.

Table 1 - Inflammatory biomarkers in depressive patients or in animal models of depression.

Inflammatory mediators	Evidence as biomarkers	Reference
↑ CRP, IL-6, IL-1β, IL-1Ra	Clinical	(162)
↑ CRP, IL-6	Clinical	(231)
↑ IL-6, TNF-α, IL-10, sIL-2R, CCL2/MCP-1, IL-13, IL-18, IL-12, IL-1Ra, sTNFR2; ↓ IFN-γ	Clinical	(180)
↑ IL-6, CXCL8/IL-8	Clinical	(182)
↑ TNF-α, IL-6	Clinical	(163)
↑ TNF-α	Clinical	(164)
↑ IL-1β	Clinical	(161)
↑ CCL2/MCP-1	Clinical	(179)
↑ IL-6, IL1β	Animal	(208)
↑ IL-6	Animal	(232)
↑ IL-6, TNF-α, CCL2/MCP-1	Animal	(233)
↑ IL-1α, ↓ CRP	Animal	(234)
↑ IL-1β	Animal	(235)

2. Non-coding RNAs

Next-generation sequencing platforms, such as RNA sequencing (RNA-seq), revealed thousands of new transcripts and opened the door to a comprehensive study of the nonprotein-coding transcriptome. The paradigm of RNA as a mere intermediary between DNA and protein became obsolete, when researchers were able to demonstrate that the previously called “junk” RNA could be of major importance for biologic diversity and may have key regulatory functions (236). Non-coding RNAs (ncRNAs) do not codify for proteins but can influence their expression and function by various mechanisms. NcRNAs comprise a heterogeneous group that includes, among others, short ncRNAs (microRNAs [miRNAs], small interfering RNAs [siRNAs], small nuclear RNA [snRNAs], small nucleolar RNAs [snoRNAs]), long ncRNAs (lncRNAs) and infrastructural RNAs (ribosomal RNAs [rRNAs] and transfer RNAs [tRNAs]) (237). In the next section, the biogenesis and function of miRNAs, their use as biomarkers and/or therapeutical targets, as well as their role in inflammation and brain diseases, will be explored in greater detail.

2.1. miRNAs biogenesis and function

microRNAs (miRNAs) are single-stranded small ncRNAs typically 15-25 nucleotides long, that can regulate gene expression at a post-transcriptional level and play a central role in homeostasis and disease-associated mechanisms (238). In 1993, joint efforts of Ambros’s and Ruvkun’s groups resulted in the discovery of the first miRNA in the *C. elegans*, *lin-4* (239, 240). Since then, the field of molecular biology was revolutionized, and miRNAs rapidly became an exciting topic of research.

The biogenesis of miRNAs ranks as one of the fastest among transcripts, often occurring within minutes (241). Half of all miRNAs annotated in miRbase (www.mirbase.org) are produced from intergenic non-coding pri-miRNA transcripts, independently of the host gene and regulated by their own promoters, while the remaining are excised from the introns of protein-coding transcripts (242). The biogenesis of miRNAs can occur through canonical and non-canonical pathways.

The canonical pathway is the dominant pathway by which miRNAs are processed. Most miRNAs originate from long RNAs called primary miRNAs (pri-miRNAs) that are regulated by conventional transcription factors and transcribed by RNA polymerase II. Pri-miRNAs form a hairpin structure and are cleaved into precursor miRNAs (pre-miRNA) by the RNase III enzyme Drosha, in a complex with RNA-binding protein (RBP) DiGeorge syndrome chromosome region 8 (DGCR8). The pre-miRNA is transported into the cytoplasm by the

nuclear export factor exportin 5 and further processed into a 15- to 25-nucleotide double-stranded RNA by the RNase III enzyme Dicer, which is bound to trans-activation-responsive RNA-binding protein (TRBP). This duplex miRNA is then incorporated into the RNA-induced silencing complex (RISC) consisting of Dicer, TRBP and Argonaute 2 (Ago2). Subsequently, one strand of the mature miRNA is retained in the RISC assembly, whereas the other strand is often degraded. Once loaded into the RISC, the mature miRNA associates with target mRNAs and acts as a negative regulator of gene expression by promoting translation inhibition or mRNA degradation. In this process, Ago2 is the catalytic centre of the RISC, responsible for cleaving the mRNA (243) (Figure 3). In mammals, the predominant mechanism is translation inhibition; however, targeted genes that are strongly downregulated at the protein level often show reduced mRNA levels (244), suggesting that mRNA destabilization also gives a major contribution to gene silencing.

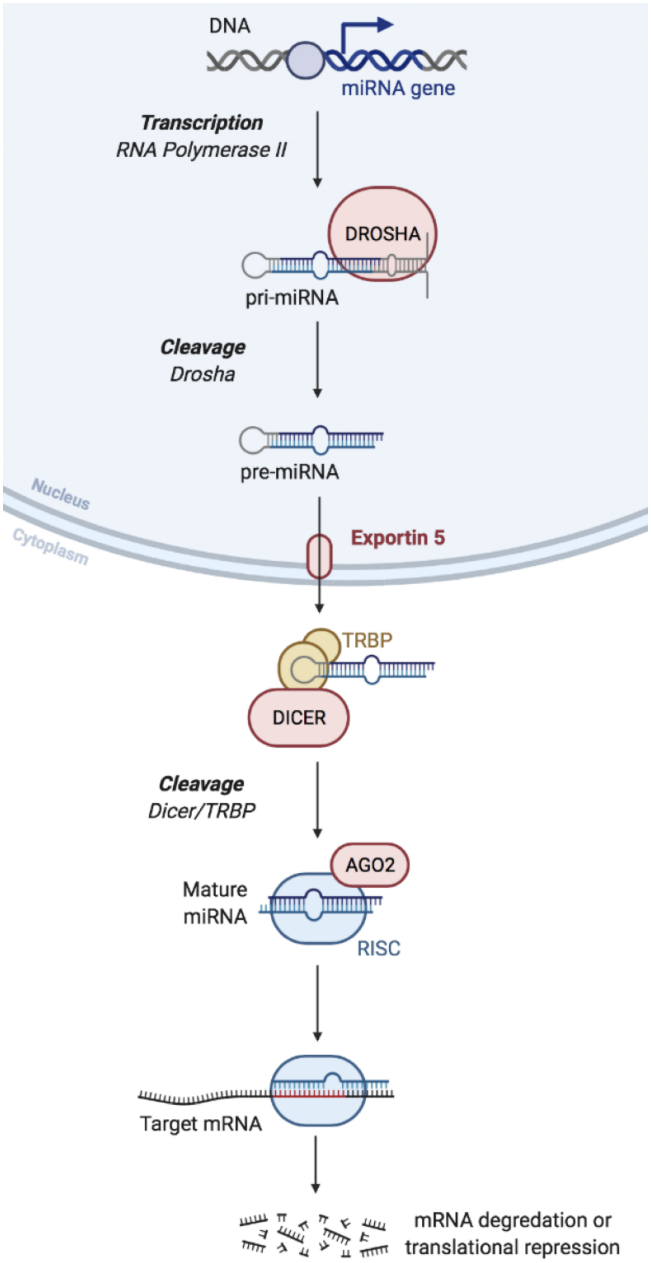


Figure 3 – Mechanism of miRNA biogenesis. In the nucleus, a primary miRNA is transcribed from the genome and processed by Drosha. Then, the precursor miRNA is exported to the cytoplasm where it is further processed by Dicer and TRBP. Mature miRNA is then incorporated into the RISC complex where it associates with target mRNAs and acts as a negative regulator of gene expression by promoting mRNA degradation or translation inhibition.

Alternatively, a residual percentage of miRNAs, can be produced by non-canonical pathways. For instance, mirtrons are intron-derived miRNAs released from their host transcripts after splicing. In case the intron has the appropriate size to form a hairpin resembling a pre-miRNA, it can bypass Drosha and be further processed by Dicer (245). Drosha-mediated processing can be also bypassed when small RNAs, derived from endogenous short hairpin RNAs, are generated directly through transcription (246). In

addition, miRNAs may be originated from other non-coding RNAs, such as tRNAs, snoRNAs or small nuclear RNA-like viral RNAs, without Drosha processing (247). Although most alternative miRNA pathways exempt Drosha, biogenesis of these small RNAs still depends on Dicer. The exception is miR-451 that does not require Dicer and instead involves the catalytic activity of Ago2 (248, 249). Pre-miR-451 stem-loop structure is too short to be cleaved by Dicer and is directly loaded onto Ago2 for maturation (248, 249).

A mature miRNA typically regulates gene expression via an association with the 3' untranslated region (UTR) of a mRNA with partially complementary sequence, although emerging evidence suggests that miRNAs may also target 5'UTRs or exons and may potentially undergo base pairing with regulatory DNA sequences to regulate transcription (250). Once miRNA bind to a 3' UTR, the degree of mRNA degradation and/or translational repression is affected by multiple mechanisms, including the overall complementarity between the miRNA and target mRNA, the secondary structure of the adjacent sequences, the distance of the miRNA binding site to the coding sequence of the mRNA, and the number of target sites within the 3' UTR (251). Contrary to siRNAs that perfectly bind to their mRNA targets with 100% complementarity, miRNAs imperfectly bind to mRNAs, with a minimum binding requirement of nucleotides 2-8 of the miRNA, referred as "seed" region. Therefore, miRNAs with high sequence homology and identical seed regions are commonly grouped into miRNA families that are likely to target similar sets of mRNAs (252).

According to mirBase v22, human genome encodes over 2600 mature miRNAs, each one of them can potentially target several mRNAs (253). On the other hand, most 3' UTRs contain potential binding sites for a large number of individual miRNAs, allowing redundancy or cooperative interactions between various seemingly unrelated miRNAs (252). The targets of some miRNAs can modulate the expression of additional miRNAs or groups of miRNAs, generating positive or negative feedback loops. miRNA maturation seems to be post-transcriptionally regulated in a sequence-specific manner, potentially explaining why genetically clustered and co-transcribed miRNAs are often expressed at different levels (254). During the last decade, nomenclature of the miRNAs has evolved. For that reason, a brief description of the most commonly used nomenclature is presented in Table 2.

Although the main role of miRNAs in mammals is intracellular gene regulation, they also play an important role in intercellular signaling. Cell free miRNAs, also known as circulating or extracellular miRNAs, can be found in body fluids such as blood, plasma, serum, urine, saliva and cerebrospinal fluid, and exhibit remarkable stability despite the presence of intense RNase activity in these fluids (255). A possible explanation is that cell free miRNAs can be packed into protein complexes of Ago2, nucleophosmin 1 (NPM 1) and high-density lipoprotein (HDL), or encapsulated in extracellular vesicles, which protects them from RNase activity (256-258). miRNAs can be also found in the extracellular space as result of

tissue damage, apoptosis or necrosis. Based on the aforementioned characteristics, miRNAs have been driving increased interest as potential biomarkers for disease diagnosis, prognosis and prediction of therapeutic responses or as therapeutical targets.

Table 2 - Nomenclature of miRNAs.

Nomenclature format	Description
***mir* or miR**	Genes that encode miRNAs, primary transcripts of miRNAs, and stem-loop precursor miRNAs are all named using the italicised prefix 'mir'. Mature miRNAs are named using the non-italicised prefix 'miR'.
***mir-X* or miR-X**	With the exception of a few early miRNAs, unique identifying numbers are sequentially assigned, depending on when they are first published, for example, mir-31 or miR-31.
mir-Xa, mir-Xb, …	Similar miRNA sequences within a species are distinguished by a lettered suffix, for example, mir-181a and mir-181b. However, this does not imply shared targets or functions.
mir-X-1, mir-X-2, …	Identical miRNA sequences within a species are distinguished by a numerical suffix, for example, mir-7-1 (chromosome 9), mir-7-2 (chromosome 15) and mir-7-3 (chromosome 19), can all produce identical mature miRNAs.
miR-X-5p or miR-X-3p	The latest convention is to name mature miRNAs by the arm of the pre-miRNA from which they are derived, regardless of their abundance - those from the 5' arm are named miR-X-5p and those from the 3' arm as miR-X-3p.
hsa-miR-X, mo-miR-X, …	All of the above naming conventions can be preceded by a three-letter code which identifies the species the miRNA is from, for example, hsa = homo sapiens; rno = rattus norvegicus.

2.2. miRNAs as biomarkers

Typically, biomarkers are defined as molecules, usually proteins (e.g., aminotransferases (ALT and AST), or prostate-specific antigen (PSA)), that can be objectively measured and evaluated from body fluids or tissues and distinguish normal biological from pathogenic processes or pharmacological responses (259).

For many years the use of RNA molecules as biomarkers was discouraged because they were thought to be unreliable markers, due to the abundant presence of nucleases, particularly in circulation, which could have implication in their stability. However, this idea was progressively dismissed as it was uncovered that miRNAs were stable in fluids and samples of fixed tissues (260). In 2004, Takamizawa *et al.* were the first to highlight the prognostic value of miRNAs by showing that, independently of disease stage, lung cancer patients presenting low let-7 expression levels have a significantly shorter survival after potentially curative resection (261). In 2005, a unique miRNA signature, composed of 13 miRNAs, was found to be associated with prognostic factors and disease progression in chronic lymphocytic leukaemia (262). In this study, Calin and colleagues showed that miRNA expression profiles could be used to distinguish normal from malignant B cells and were associated with prognostic factors such as ZAP-70 expression and mutational status of IgV (H) (262). Nonetheless, the first reports addressing the utility of miRNAs as diagnostic tools in biological fluids were only published in 2008. First, Chim *et al.* demonstrated the existence of placental miRNAs in maternal plasma (263) and later that year, Lawrie and co-workers detected elevated levels of tumour-associated miRNAs in the serum of patients with diffuse large B-cell lymphoma (264). Ever since, the potential use of miRNAs as biomarkers has been explored for numerous pathologies.

In fact, a biomarker based on miRNA levels has several advantages. In addition to their stability in body fluids, miRNA levels can be measured rapidly and accurately, using robust polymerase chain reaction (PCR) and sequencing technologies that enable the analysis of billions of sequences in a single run with very high precision (265). Another advantage of miRNAs lies in their potential for being used as multimarker panels composed of numerous miRNAs for accurate diagnosis, guided treatment and evaluating treatment response (266). Furthermore, PCR-based amplification enables the measurement of very low amounts of miRNAs in a small sample volume (267).

Currently, the clinical utility of miRNAs as diagnostic/prognostic biomarkers has been demonstrated in several types of pathologies, especially in cancer and in age-related diseases (268). To this extent, companies have been working in the development of miRNA-based diagnostic panels, with some products being already available in the market.

In the field of cancer, miRview™ mets, launched by Rosetta Genomics and Precision Therapeutics in 2012, was the first miRNA panel available for diagnostic purposes, specifically for cancers of unknown or uncertain primary origin (CUP). This second-generation assay is able to identify 42 different tumour types using a microarray that measures the expression levels of 64 miRNAs, with a precision of 90% (269). In 2016, Interpace Diagnostics and Asuragen released a personalized medicine strategy for the diagnosis of thyroid and pancreatic cancer. By combining ThyraMIR®, a miRNA classifier, and ThyGeNEXT®, an oncogene panel for thyroid cancer stratification, Interpace Diagnostic claims a Negative Predictive Value of 94%, a Positive Predictive value of 74% and a reduction of 85% of unnecessary surgeries (270). The ThyraMIR® classifier includes the quantification of 10 miRNAs: miR-29b-1-5p, miR-31-5p, miR-138-1-3p, miR-139-5p, miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375 and miR-551b- 3p (270). Although both tests are not FDA approved yet, Interpace Diagnostics is CLIA certified, CAP accredited and covered by major insurance companies that have agreed to include ThyGeNEXT®/ ThyraMIR® combined tests in the health plans of their 5.3 million beneficiaries in the U.S..

Funded in 2013, TAmiRNA is the European Leader in miRNA diagnostics. The Austrian R&D company develops and offers validated miRNAs panels as additional tools for the diagnostic of age-related disorders. OsteomiR® is their lead product intended to provide the risk of a first fracture in female patients with postmenopausal osteoporosis and type-2 diabetes (271). The osteomiR® enables individual analysis of 19 miRNA biomarkers in human serum sample, allowing the calculation of a fracture-risk score for preventive therapy and treatment follow-up (272-274). TAmiRNA also proposes the ThrombomiR® panel (12 miRNAs) to assess cardiovascular diseases risk. The thrombomiR® kit provides an *in vivo* measure of platelet function by detecting miRNAs secreted from platelets during their activation that can be detected in plasma and serum (275, 276).

Currently, these are the only miRNA-based products for diagnostic purposes currently available in the market. Others, such as CogniMIR™ (DiamiR) for early detection of mild cognitive impairment and Alzheimer's disease (277, 278) and Simoa® (Quanterix/DestiNA Genomics) for detection of miR-122 as a liver toxicity marker (279), are now in pre-clinical phases or already in phase 1 clinical trials. Of note, Simoa® is a patented unique PCR-free, chemical-based technology for the detection and quantification of nucleic acids, without prior isolation from serum or plasma (280).

It is evident that this field of research has an incredible potential. However, it is still in its early stages, and the most promising findings often lack reproducibility (281). Particularly, the use of blood-based miRNA biomarkers, being it from plasma, serum or peripheral blood mononuclear cells (PBMCs), needs deeper research and protocol harmonization (281). In

order to maximize the clinical application of newly discovered disease-associated miRNAs, the scientific community needs to develop standardized procedures for collection, transport, storage and sample processing, housekeeping miRNAs used for normalization that may vary between tissues, as well as harmonized data analysis procedures for the diversity of technological methods used.

2.3. miRNAs as therapeutical targets

As the vast majority of human diseases involve the deregulation of multiple genes, modern pharmacology aims to regulate several targets in a multi-pronged approach. miRNAs' unique expression patterns and ability to modulate mRNA levels of a large number of target genes, increases their desirability as therapeutical tools (268). miRNAs regulation often occurs through modulation of genes in a tissue or cell-specific manner. Additionally, miRNAs have been found to be aberrantly expressed in several pathologies, either over- or under- expressed in different diseases (282, 283). Therefore, miRNA therapeutics aims to restore the levels of a particular miRNA. The advances in RNA chemical modifications and delivery systems have led to successful antisense oligonucleotides (ASO) and siRNA therapeutics that have been lately applied to miRNAs-based drugs (284). When miRNAs downregulation is associated with the development or progression of disease, chemically synthesized double-stranded miRNA mimics are often used to restore the mature miRNA levels in tissue/cells. (285). An alternative approach is the delivery of synthetic short-hairpin RNAs (shRNAs) that are incorporated and processed by the cell in a controlled manner into mature miRNAs (285). On the contrary, antagomiRs and miRNA sponges are usually used to suppress the function of miRNAs that are overexpressed in diseases. Antagomirs are chemically modified oligonucleotides that bind specifically to particular miRNAs, while miRNA sponges are small synthetic RNAs that bind to multiple miRNAs that have the same 'seed region' (286). Anti-miRNA antisense oligonucleotides (AMOs), miRNA decoys, or circularized anti-miRNAs, most of which based on antisense molecules, are additional strategies used to bind and sequester miRNAs from their natural targets (284).

In 2005, Krutzfeldt *et al.* reported for the first time the use of antagomiRs as efficient and specific silencers of endogenous miRNAs in mice (287). Systemic administration of antagomirs against miR-16, miR-122, miR-192 and miR-194 resulted in a marked reduction of the corresponding miRNA levels in several tissues (287). Since then, many preclinical studies have been focusing on the therapeutical potential of miRNAs. However, despite several miRNAs' studies showing great treatment promise, some obstacles remain in the field. miRNA therapeutics are less advanced than the miRNA products for clinical diagnosis

of diseases. Currently, the most advanced projects are in phase 2 clinical trials and are mainly based on miRNA mimics or antagomiRs.

SPC3649 or Miravirsen (Santaris Pharma/Roche) is an antagomir targeting miR-122 currently undergoing multiple phase 2 clinical trials (NCT01200420 and NCT02508090). miR-122 is a liver-specific miRNA with an important role in the life cycle of hepatitis C virus (HCV) (288). Miravirsen is composed of locked nucleic acid (LNAs) ribonucleotides interspaced throughout a DNA phosphorothioate sequence complementary to mature miR-122 (289, 290). The LNA modifications endow the drug with high affinity for its target and provide a natural mechanism of accumulation in the liver, dispensing a special delivery strategy (289, 290). In phase 2a clinical trials, Miravirsen monotherapy in patients with genotype 1 (gt1) chronic HCV infection was associated with dose-dependent reductions in HCV RNA that were prolonged following dosing, while plasma levels of other miRNAs were not significantly affected by antagonising miR-122 (291, 292).

MRG-201 or Remlarsen (miRagen Therapeutics) was designed to mimic the activity of miR-29 that decreases the expression of collagen and other proteins involved in extracellular matrix deposition and scar formation (293). The miR-29 family (miR-29a/b/c) is constantly downregulated in fibrotic diseases (294, 295) . A phase 2 clinical trial is currently underway to determine if intradermal injection of Remlarsen can limit the formation fibrotic tissue in pathological cutaneous fibrosis (NCT03601052). Based on the promising results of phase 1 clinical trial (NCT02603224), miRagen is now also developing MRG-229, a second-generation mimic of miR-29, for the treatment of idiopathic pulmonary fibrosis. In parallel, miRagen is actively developing MRG-106 or Cobomarsen, a LNA antagomir that targets miR-155, which is found at abnormally high levels in malignant cells of several blood cancers (296). Currently, Cobomarsen is involved in phase 1 (NCT02580552) and phase 2 (NCT03713320) clinical trials, to treat certain types of T-cell lymphoma and leukaemia. Also, in the field of cancer and after a successful phase 1 clinical trial, ENGeneIC is now heading to a phase 2 clinical trial (NCT02369198) to test the efficacy of Mesomir, a miRNA mimic that aims to replace miR-16, a tumour suppressor that is reduced in malignant pleural mesothelioma (297, 298).

On another hand, Abivax developed ABX464, an oral, small molecule that upregulates the production of a unique RNA splicing product and anti-inflammatory agent, miR-124. ABX464's mechanism of action has shown promise in clinical trials (NCT03093259 and NCT03368118) in its ability to bring patients into remission and heal inflammatory lesions in cases with moderate-to-severe UC and CD, who had failed immunomodulators, anti-TNF-α, vedolizumab and/or corticosteroids (299, 300).

2.4. miRNAs in inflammation and innate immunity

During the last years, miRNA mechanism of action, targets and contribution to immune cells function have been examined. Known as “fine-tuners” of immune responses, miRNAs play pivotal roles in cell development, differentiation and homeostasis. As such, miRNA interaction with transcription factors and signaling molecules greatly influence a magnitude of an inflammatory response (301). Certain miRNAs amplify inflammation by repressing inhibitors of the response, whereas others serve in important negative feedback loops in the immune system (302, 303). Many miRNAs are co-regulated with protein-coding genes during the inflammatory response. On one hand, miRNAs levels can be regulated at a transcriptional level as part of the inflammatory transcriptional program itself (304). On the other hand, several proteins that are induced during an inflammatory response, can regulate the processing of miRNAs by conditioning the expression or function of key molecules such as Drosha and/or Dicer (305, 306).

miRNA mechanisms of action in the immune system take place at distinctive regulatory levels. Firstly, miRNAs regulate time-sensitive aspects of gene regulation. As opposed to protein transcriptional repressors, inflammation-induced miRNAs (inflammiRs), do not require neither translation nor nuclear translocation to repress their targets, allowing them to act in a shorter time frame (255). Commonly referred as early response inflammiRs, they play an important role in innate immune responses where a short time frame is critical to begin the response. Secondly, miRNAs may have distinct functions in different cell types. Although the mechanism of action is the same, the transcriptional program of a given cell type limits the number of relevant targets for a given miRNA. Hence, a cell undergoing massive transcriptional activation during inflammation will have a different set of targets regulated by a given miRNA (307). And thirdly, miRNAs regulate gene expression by incomplete repression of their targets. As opposed to transcription factors that act as ‘switches’, miRNAs have the capacity to “buffer" gene expression patterns by causing small changes in many genes (308). Nonetheless, miRNAs can also drastically change cellular gene expression patterns by targeting key transcription factors.

As mentioned before, the initiation, spread and resolution steps of an inflammatory response are subject to both positive and negative regulatory events mediated by miRNAs. The positive feedback initiates a cascade of molecular events that serve to combat against pathogens and successfully repair the tissue (302). The negative feedback, which is activated only during severe/high-grade inflammation is vital for preventing potential damaging end-stage processes and maintaining tissue homeostasis (309). Previous work from our group in an injured bone model, shows this temporal regulation by the let-7 family members, that appear down-regulated at day 3, during the acute inflammatory phase, and

upregulated at day 14, during the resolution phase (310). When an inflammatory response takes place, the coordinated activation of multiple signaling pathways, regulate the expression of both pro- and anti-inflammatory mediators, such as miRNAs (301). While some miRNAs function relies specifically in favouring pro- or anti-inflammatory processes, some others can have a dual role depending on the immune response stage. These miRNAs are usually found upregulated in response to cell priming and activation, potentiating an initial response. Then, in turn, they are able to suppress key signaling molecules in order to control and resolve the inflammatory response (307). When this "on-off" function fails, inflammatory responses may become chronic, resulting in additional complications. Elevated systemic chronic inflammation can lead to a basal low-grade constitutive activation of various signaling pathways, which results in a weakened acute response to multiple stimuli (311). In this section the function and involvement of several miRNAs in inflammatory processes and inflammation-related diseases will be further detailed (Table 3).

miR-9

Similar to other miRNAs, miR-9 is acts as a negative feedback player and contributes to inflammation resolution. miR-9 expression is induced by myeloid differentiation primary response 88 (MyD88)-activating TLR agonists as well as by proinflammatory cytokines TNF-α and IL-1β, in a natural mechanism to operate a feedback control of the NF-κB-dependent response (312). miR-9 targets *NFKB1* mRNA, which encodes for NF-κB p50 subunit, that has an important role in transactivation of the NF-κB p65 subunit (312). Additionally, miR-9 targets Janus Kinase 1 (JAK1) and inhibits activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome in THP-1 derived macrophages (313).

miR-21

miR-21 is abundantly expressed in many tissues, and recent studies have revealed an essential role in the resolution of inflammation by a negative feedback of inflammatory pathways. miR-21 expression is enhanced in activated immune cells and, accordingly, it has been found upregulated in osteoarthritis (OA), allergic airway inflammation and heart disease (314-316). As opposed to miR-155, that is induced immediately after NF-κB activation, miR-21 is produced in a delayed time frame in order to act as a negative feedback regulator of the immune response (317). miR-21 was shown to target programmed cell death 4 (PDCD4), a pro-inflammatory molecule, reducing the production of IL-6 and TNF-α, while favouring IL-10 expression, implying an anti-inflammatory effect (318).

miR-124

miR-124 is known as the most abundant miRNA in the brain, playing a key role in neurogenesis, but has recently emerged also as critical negative regulator of inflammation. miR-124 targets key molecules involved in Signal Transducer and Activator of Transcription (STAT) and TLRs pathways, including STAT3, TLR6, MyD88 and TNF receptor-associated factor 6 (TRAF6), directly impacting the production of IL-6 and TNF-α (319-321). miR-124 has been found downregulated in pathologies with uncontrolled inflammation, such as in RA, severe pulmonary hypertension, paediatric intestinal failure, and paediatric active UC (319, 322-324).

miR-125

Recent studies have shown that miR-125 family plays an important role in promoting inflammation resolution. miR-125a regulates phagocytic and bactericidal activities of macrophages, by inhibiting LPS-mediated pro-inflammatory phenotype, in favour of IL-4-induced inflammation resolution phenotype, via targeting of Kruppel Like Factor 13 (KLF13) (325). In addition, miR-125b regulates TNF-α production by targeting the 3'UTR of its transcript (326). Interestingly, miR-125b has been found downregulated after TLR-priming, in a mechanism that appears to be required to stabilize TNF-α production (326, 327). Plasma miR-125a is decreased in CD patients with active disease status and negatively correlates with disease severity and inflammatory cytokines levels (328). miR-125a was also found downregulated in systemic lupus erythematosus (SLE) patients, contributing to elevated inflammatory chemokine RANTES/CCL5 levels and KLF13 (329).

miR-132

A dual role in mediating inflammatory responses has been attributed to miR-132. On one hand, miR-132 is initially upregulated in response to LPS-driven macrophage activation and has been associated with increased IL-8 and CCL2 secretion, via targeting silent information regulator 1 (SIRT1) (330, 331). On the other hand, miR-132 serves as “buffer” to control inflammation in the nervous system. miR-132 targets acetylcholinesterase (AChE) that hydrolyses acetylcholine, a neurotransmitter of the cholinergic anti-inflammatory pathway (332). So, an increase in miR-132 levels in response to TLR stimulation results in the repression of AChE and increased acetylcholine-mediated negative regulation of TLR-induced signals (332). In agreement, miR-132 levels were found to be higher in inflamed compared with quiescent intestinal biopsies from patients with IBD. Correspondingly, AChE activity was significantly lower in patients with IBD suffering from moderate–severe disease as compared with healthy controls or patient with IBD presenting low disease severity (333).

miR-145

miR-145 have been frequently associated with anti-inflammatory actions. miR-145 attenuates pro-inflammatory cytokines production by targeting key molecules of TLR signaling such as the toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP), the mothers against decapentaplegic homolog 3 (SMAD3), TRAF6 and CD40 (334-336). In OA, miR-145 was shown to be directly repressed by NF-κB p65 and negatively correlated with TNF-α secretion. Its overexpression led to a restrained production of several TNF-α-triggered matrix-degrading enzymes, by supressing mitogen-activated protein kinase kinase 4 (MKK4) (337).

miR-146

The miR-146 family comprises miR-146a and miR-146b, that are expressed in response to pro-inflammatory stimuli, acting as important negative regulators of inflammatory responses. miR-146a and miR-146b expression is frequently induced by NF-κB, which in turn targets TRAF6 and IL-1 receptor-associated kinase 1 (IRAK1), two important components of the NF-κB pathway, resulting in a negative feedback loop (338, 339). Importantly, miR-146a negatively regulates the IFN response, the adaptive immunity by targeting adaptor protein (AP)-1 activity and IL-2 expression, as well as immune cell activation and cytokines production (340, 341). miR-146a expression is upregulated in pathologies associated with high-grade increased inflammation, such as RA, OA and psoriasis (282). However, in pathologies with baseline low grade chronic inflammation, such as hypertension and obesity, miR-146a has been found downregulated (342, 343).

miR-155

miR-155 is known as a master regulator of innate immune responses. miR-155 has been recurrently found upregulated in inflammation-related diseases, such as rheumatoid arthritis, inflammatory bowel disease, atopic dermatitis and atherosclerosis (282). This miRNA is rapidly increased in response to NF-κB activation, targeting the suppressor of cytokine signaling 1 (SOCS1) and the inositol polyphosphate-5-phosphatase (SHIP1), which leads to increased production of pro-inflammatory and cytotoxic mediators such as TNF-α, IL-1β and nitric oxide (NO) (344). Although miR-155 mainly exerts pro-inflammatory functions it was recently shown that an increase of miR-155 may relieve chronic inflammation by a negative feedback loop (345, 346). At the initial stages innate immune responses, inflammatory factors such as TNF-α are overexpressed leading to NF-κB activation, thus resulting in a sharp increase in the miR-155 levels. miR-155 in turn was shown to suppress the production of TNF-α contributing to the resolution of the inflammatory response during atherosclerosis-associated foam cell formation (345).

miR-181

Accumulating evidence indicates an important role for the miR-181 family in inflammation. miR-181a regulates inflammatory responses in monocytes and macrophages, by targeting IL-1α and inhibiting the production of classical pro-inflammatory cytokines and reactive oxygen species (ROS) (347). In addition, miR-181a was identified as a negative regulator in high mobility group box-1 protein (HMGB1)-induced immune responses by targeting TNF-α mRNA, in dendritic cells (348). In turn, miR-181b was found downregulated in critically ill patients with sepsis compared with control intensive care unit subjects. miR-181b was shown to have an essential role in regulating endothelial cell activation and immune cell homeostasis, by targeting importin-α3 , a protein required for nuclear translocation of NF-κB, in endothelial cells *in vitro* and *in vivo* (349). Moreover, miR-181 family seems to be important in neuroinflammation. miR-181-b, -c and -d were found reduced in the cerebral cortex of LPS-injected mice and the knockdown of miR-181b and -c in astrocytes enhanced LPS-induced production of TNF-α, IL-6, IL-1β, and IL-8, while their overexpression resulted in a significant increase of IL-10 (350). Also, miR-181c was shown to control microglia-mediated neuronal apoptosis by suppressing TNF-α (351).

miR-223

miR-223 is another TLR-responsive miRNA, up-regulated by NF-κB activation. miR-223 acts as a negative feedback regulator of inflammation, by targeting key molecules involved in inflammatory responses, such as the inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα), STAT3 and PBX/knotted 1 homeobox 1 (Pknox1) (352-354). In addition, miR-223 targets NLRP3, decreasing caspase-1 activation and subsequent processing of IL-1β (355). miR-223 was found increased in intestinal biopsies from patients with active IBD and in preclinical models of intestinal inflammation (356). However, complete ablation of miR-223 in mice produced exacerbated myeloid-driven colitis with a marked increase in tissue inflammation, particularly enhanced NLRP3 inflammasome activity with elevated IL-1β production (357).

let-7

let-7 was the first miRNA to be discovered in human cells in 2000 (358). Since then, let-7 family is among the most studied miRNAs and are known to regulate developmental timing and cell proliferation, and to mediate immune responses and promote inflammation resolution. let-7 function as efficient fine-tuners of gene expression to balance immune cell responses, which when left unchecked, can have physiological or pathological effects. Specifically, let-7b, -7e and -7i were shown to target TLR4, thereby decreasing NF-κB-driven inflammation, while let-7a and -7b directly target proinflammatory cytokine IL-6 (359-

361). Interestingly, let-7 miRNAs themselves are negatively regulated by TLR/NF-κB (362), reinforcing their involvement in the inflammatory response and potential as therapeutical targets. In macrophages, let-7c overexpression was shown to promote M2 polarization and to regulate bactericidal and phagocytic activities, by targeting CCAAT enhancer binding protein gamma (C/EBP-δ) (363). The protective role of let-7 miRNAs was also shown in *Mycobacterium tuberculosis* (Mtb). By targeting A20, a NF-κB inhibitor, let-7f enhanced the production TNF-α and IL-1β drastically reducing Mtb survival (364).

Table 3 - miRNAs in inflammation and innate immunity.

miRNA	Targets	Effect
miR-9	NF-κB1, JAK1	Negative regulator of TLR and NLRP3 signaling
miR-21	PDCD4	Anti-inflammatory effect; negative regulator of TLR signaling
miR-124	STAT3; TLR6; MyD88; TRAF6	Negative regulator of TLR and STAT signaling
miR-125a^{+}, miR-125b$^{\#}$	KLF13$^{+;}$ TNF-α$^{\#}$	Promote inflammation resolution
miR-132	SIRT; AChE	Enhances inflammation; serves as “buffer” to control inflammation in the nervous system
miR-145	TIRAP; SMAD3; TRAF6; CD40; MKK4	Negative regulator of TLR signaling
miR-146a, miR-146b	TRAF6; IRAK1	Negative regulator of TLR signaling
miR-155	SOCS1; SHIP1; MyD88; IKKε	Enhances inflammation; negative feedback regulation
miR-181a^{+}, miR-181b$^{\#}$	IL-1α^{+}; TNF-α^{+}; KPNA3$^{\#}$	Diminish inflammation
miR-223	IKKα; STAT3; NLRP3	Negative feedback regulator of inflammation
let-7 family	TLR4; C/EBP-δ; A20	Downregulate inflammatory signaling

$^{+,\#}$ indicate the correspondent target of each miRNA.

2.5. miRNAs in CNS diseases: role in neuroinflammation and microglia activation

Over the last decade, neuroinflammation has been recognized as a common component of the pathogenesis in multiple neurodegenerative and neuropsychiatric diseases (365). The neuroinflammatory process is a self-defence attempt by the CNS to remove harmful stimuli (e.g., pathogens, damaged cells, or toxins) and to initiate the healthy process as an orchestrated stereotype, which contains three stages: initiation, propagation and resolution. However, uncontrolled neuroinflammation play a critical role for gradually loss of neurons by time. In turn, dying neurons prompt reactive microgliosis in order to sustain and maintain a lower grade of neuron-inflammation (366). This self-propagating cycle formed by damaged neurons and reactive microglia forces low grade chronic inflammation, which impacts normal neurocircuits favouring the progressing pathogenesis of diseases with long-lasting and non-cell autonomous manner (311).

Besides their immunity role, microglia also orchestrate several processes, critical for proper neuronal functioning. In the healthy brain, microglia are implicated in a series of physiological tasks, ranging from removal of neuronal debris to precise refinement of synaptic terminals, contributing to maturation of neural circuits (367, 368). Microglia are very dynamic, continuously surveying the parenchymal environment through highly motile ramified processes, to monitor for damage associated disease, constantly engaged in physical contacts with neighbouring cells, and, specifically, with synaptic structures (369). miR-124, the most abundant miRNA expressed in neurons, plays an important role also in microglia development (370). By targeting the transcription factor CEBPα and the cyclins CDK4 and CDK6, miR-124 is able to reduce the expression of PU.1 and its downstream target, the M-CSF receptor, restricting cellular proliferation and potentiating the differentiation of primitive macrophages to adult microglia, as well as contributing to the maintenance of a quiescent state (371).The high levels of miR-124 observed in adult microglia are believed to be a specific consequence of the CNS environment, particularly through the crosstalk with neurons. Neuronal cells can directly secrete miR-124 through exosomal shuttle vesicles or cell-to-cell contact, or indirectly, by stimulating its expression through the release of anti-inflammatory factors, such as CX3CL1 and TGF-β (370).

Microglial autophagy is a pivotal process for debris clearance, cytokine production and cellular survival. Disruption of normal autophagy flux contributes to neuronal cell death in several neurodegenerative diseases. Let-7a is a key modulator of the autophagy system, enhancing autophagy efficiency in various inflammatory responses. let-7a overexpression in LPS-treated microglia was shown to upregulate the expression of autophagy-related factors Beclin1, ATG3, and microtubule-associated protein 1A/1B-light chain 3 (LC3) (372).

A downregulation of miR-195 was shown to promote increased activation of microglial autophagy. Accordingly, inhibition of miR-195 expression suppresses neuroinflammation and neuropathic pain (via IL-1β, iNOS, and TNF-α) through 3′UTR targeting of autophagy related 14 (ATG14) (373). Moreover, miR-144 was shown to induce abnormal microglial autophagy and inflammation following intracerebral haemorrhage (ICH). miR-144 overexpression was associated with a downregulation of mTOR, an inhibitor of the NF-κB pathway. *In vivo* downregulation of miR-144, improved neuroinflammation and neurological functions in ICH mice (374). Similarly, miR-223 deficiency significantly ameliorated CNS inflammation, demyelination and the clinical symptoms of experimental autoimmune encephalomyelitis (EAE) and increased resting microglia and autophagy in brain microglial cells (375).

As referred above, alterations in the physiological state of microglia contribute to disease progression, and several genes that are dysregulated in microglia are associated with increased risk for disease. In this sense, increasing interest has been given to the role of miRNAs in microglia dysfunction and neuroinflammation, particularly those associated with neurodegenerative diseases, including Alzheimer's Disease (AD), Parkinson's Disease (PD) and Multiple Sclerosis (MS).

AD is a chronic and progressive neurodegenerative disorder that results from the loss of synapses and is characterized by the build-up of extracellular plaques of aggregated amyloid beta (Aβ) peptides and intracellular neurofibrillary tangles (NFTs) of hyper-phosphorylated tau (376). Proliferation and activation of microglia in the brain, concentrated around Aβ plaques, is a prominent feature of AD (377). While microglia-mediated clearance of accumulated Aβ protect against the incidence of AD, impaired microglial activities and altered microglial responses to Aβ are associated with increased AD risk (378). In fact, the majority of risk genes for AD are highly expressed by microglia in the brain (379). A selective subset of miRNAs including miR-34a, miR-125b, miR-146a and miRNA-155 has been found dysregulated either in the brain of AD patients or in human brain cells primary cultures when stressed with AD-relevant stressors. miR-155 was recently shown to influence fibrillar Aβ catabolism by microglia (380). Deletion of miR-155 led to an increase of Aβ in lysosomal compartments, while its overexpression decreases Aβ catabolism. In the same study, the authors found that LPS-induced microglia activation resulted in quick-expression of miR-155 and delayed miR-146a expression (380). In fact, miR-146a has been recurrently found elevated in the brain of AD patients and to be increased in response to pro-inflammatory mediators such as IL-1β, TNF-α and Aβ (381-383). This upregulation was associated with decreased levels of the glycoprotein immune repressor complement factor (CFH), suggesting that miR-146a upregulation contributes to AD pathogenesis (384, 385). However, this theory has been recently questioned as there is increasing evidences that

miR-146a rather plays a pivotal role in keeping microglial inflammatory response “in-check”, by producing fairly low levels of pro-inflammatory cytokines, presumably as an inherent defence mechanism to prevent exacerbation of neurodegenerative cascade. For instance, it is known that patients with Presenilin 2 (PSEN2) mutations develop autosomal dominant AD. miR-146a was found downregulated in PSEN2 KO microglia, while its direct target IRAK1 was found upregulated, thereby increasing NF-κB transcriptional activity (386). In line with these, miR-146a overexpression in prion disease has been associated with a downregulation of important phagocytosis and oxidative burst genes, such as iNOS and IL-1β, and to alterations in downstream mediators of the NF-κB and JAK-STAT signalling pathway (387). miR-34a was shown to target the triggering receptor expressed on myeloid cells 2 (TREM2), impairing microglial phagocytic responses that ultimately contribute to Aβ aggregation and accumulation and inflammatory degeneration (388). Finally, miR-125b has recurrently found upregulated in the brain of AD patients (382), and it is believed to be a central member of AD pathogenesis by driving deficits in phagocytosis (TREM2), innate immune signaling and chronic inflammation (IkBKG, CFH), impairments in neurotransmitter packaging and release (SYN-2), and neurotrophism (15-LOX, VDR) (389).

PD is another neurodegenerative disease of the CNS which mainly causes motor defects due to the loss of dopaminergic neurons, accumulation of inclusions within neuronal cell bodies (Lewy bodies), and increased microgliosis in the substantia nigra pars compacta (390). Lewy bodies are aggregates of insoluble protein, the major component of which is α-synuclein (α-syn), a protein normally abundant in neurons that misfolds into conformations, that promotes microglia activation and confers neurotoxicity during PD (390). Therefore, microglial morphological and functional changes produced by aberrant miRNA expression have been also observed in PD. miR-29 family was found downregulated in the serum of PD patients (391). miR29c overexpression suppresses neuroinflammation, either by reducing the levels of pro-inflammatory cytokines and by inhibiting microglia activation via targeting of nuclear factor of activated T-cells (NFAT) 5 (392, 393). Importantly, overexpression of miR-29c attenuated dopaminergic neuronal death and α-synuclein aggregation in a PD mice model (392). miR-30 family members have been also found dysregulated in PD. In post-mortem human samples, miR-30b, miR-30c-2 and miR-30d were found upregulated across different brain regions in PD patients (394, 395). Conversely, downregulated expressions of miR-30b and miR-30c in the PBMCs and downregulated miR-30a in the plasma were reported in PD patients (396, 397). Despite the discrepancies, these discoveries suggest that miR-30 may play functional roles in PD progression. In fact, delivery of miR-30e was shown to ameliorate neuroinflammation by targeting the activation of NLRP3 inflammasome and reducing inflammatory cytokines TNF-α, COX-2 and iNOS, thereby improving mice’s motor behavioural performances in a 1-

methy1-4-pheny1-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model (398). Finally, anti-inflammatory and neuroprotective roles have been attributed to the miR-7 and miR-124 in PD. These miRNAs were found downregulated in PD models (399, 400), and their overexpression repressed neuronal α-syn and microglial MEKK3/NF-κB expression, thereby limiting their associated neurotoxicity and neuroinflammation (401, 402).

MS is a chronic autoimmune disease of the CNS that as a neurodegenerative disease caused by demyelination. The neuroinflammatory component of MS results from microglia hyperactivation and infiltration of macrophages and autoreactive lymphocytes into the CNS following disruption of the blood brain barrier (BBB) (403). miR-155, found overexpressed in MS lesions, is known to promote neuroinflammation by increasing BBB permeability to peripheral immune cells and by inducing microglia activation towards a pro-inflammatory phenotype (370, 404). In addition to miR-155, miR-34a and miR-326 were also found upregulated in MS lesions and shown to target inhibitor of phagocytosis CD47, releasing macrophages from inhibitory control, thereby promoting phagocytosis of myelin (405). Recently, miR-142 was found significantly increased in the frontal white matter from MS patients and was associated with the pathogenesis of autoimmune neuroinflammation by influencing T cell differentiation (406).

In contrast, the understanding about the role of miRNAs in microglia function and neuroinflammation in psychiatric disorders is less advanced. Nonetheless, during recent years first steps have been taken to explore the role of the immune system in pathophysiology of these disorders as well as to understand how miRNAs may appear dysregulated.

Schizophrenia (SZ) is a psychiatric disorder affecting up to 1% of the population in their lifetime, whose underlying cause remains to be elucidated. Several biological factors have been proposed, including abnormalities in oligodendrocytes, NMDA signaling and dopaminergic transmission, however, is neuroinflammation that has recently prompted more attention as a contributor to the pathogenesis of SZ (407). Clinical studies, including neuroimaging, peripheral biomarkers and randomized control trials, have suggested the presence of neuroinflammation in schizophrenia. A study evaluating the prefrontal cortex (PFC) of SZ patients reported increased mRNA levels of NF-κB, IL-1β, IL-6 and IFN-β, that were associated with an increase of viral restriction factor interferon-induced transmembrane protein (IFITM) and a decreased of its inhibitor Schnurri-2 (408). Moreover, a significant percentage of studies evaluating microgliosis in SZ have reported increased expression of major histocompatibility complex (MHC) II molecules, HLA-DR, in SZ patients across different brain regions (409, 410), even more evident in suicide victims (411). Despite that, there is still some variability across studies (412), that can be partially explained by multiple factors including brain region, source of the brain, diagnosis, age of

onset and the presence of suicide victims in the cohort. In fact, this variability seems to also apply to miRNA expression analysis in this group of patients. For instance, the miR-132/212 cluster have been found dysregulated in post-mortem brain samples of SZ patients, but results are contradictory between studies. Kim *et al.* analysed post-mortem tissue from the dorsolateral PFC (DLPFC) and found increased levels of miR-132 and miR-212, as well as miR-7, miR-34a, miR-154 and miR-544 (413). However, other studies report a downregulation of both miR-132 and miR-212 in the brain of SZ patients (414, 415). On the contrary, miR-181b has been found upregulated in the temporal cortex, DLPFC, as well as in PBMCs of SZ patients (416, 417). Besides miR-181b, a recent meta-analysis evaluating the diagnostic value of blood-derived miRNAs for SZ also found miR-21, miR-34a, miR-137 and miR-195 upregulated and miR-346 downregulated in the PBMCs of SZ patients (418). Despite these findings, the disease-associated function of these dysregulated miRNAs should be further studied, particularly their role in neuroinflammation and its deleterious effects in SZ.

Bipolar disorder (BD) is a mood disorder characterized by recurrent episodes of elevated (mania or hypomania), depressed, or mixed mood. Several lines of evidence indicate that the neuroimmune system and neuroinflammation play a role in the pathophysiology of BD. A meta-analysis of blood cytokine alterations in BD revealed that levels of IL-1β, IL-4, IL-6, IL-10, sIL-2R, sIL-6R and sTNF-R1 were significantly increased in chronically ill patients with euthymic BD compared with controls (419). Increased levels of microglia markers in the post-mortem frontal cortices and increased binding of the PK11195 positron emission tomography (PET) ligand, indicative of neuroinflammation, in the right hippocampus of BD patients have also been reported (420, 421). Studies of miRNA expression in BD have also shown significant alterations in post-mortem brain tissue from affected subjects. Moreau *et al.* evaluated the expression profiles of 435 miRNAs in post-mortem brain samples from individuals with SZ and BD, and found that of 19% of the miRNAs analysed exhibited positive evidence of altered expression due to a diagnosis of SZ or BD. Both conditions were associated with reduced miRNA expression levels, with a much more pronounced effect observed for BD (422). Nonetheless, most of the findings regarding miRNA differential expression in BD, have not been replicated between independent studies. For instance, miR-34a was reported to be downregulated in the cingulate cortex but upregulated in the cerebellum (423, 424), while miR-106b initially found downregulated in the PFC, was later reported to be upregulated in the same region (422, 425). Despite substantial heterogeneity, some miRNAs have appeared dysregulated in the same trend in multiple studies, including miR-29c and miR-132 that have been found upregulated (426). Some of these miRNAs, have also been associated with SZ suggesting potential common pathways that are affected by miRNAs in these psychotic syndromes (426). As in the case of SZ, further investigation

of these more robust miRNAs and their target genes should provide insight into the role they play in influencing the development of BD, hopefully potentiating their use as biomarkers or therapeutical targets.

2.6. miRNAs in depression

The contribution of microglia activation and neuroinflammation to the pathophysiology of depression have been recently explored and was detailed in section 1.2. Although not studied as deeply as in neurodegenerative diseases, several miRNAs have been also reported to be dysregulated in depression. Post-mortem studies evaluating the expression profile of miRNAs have focused on brain regions known to be directly related to depression symptoms (e.g., PFC, amygdala, and hippocampus). Smalheiser *et al.* were the first to report changes in the expression profile of miRNAs in depressed individuals (427). This study, carried out in PFC samples of non-medicated depression patients that committed suicide and healthy controls, found an overall miRNA expression downregulation in the PFC of depressed suicide subjects (427). However, a subsequent study performed in a similar experimental setup found an upregulation of miR-139-5p, miR-195, miR-320c and miR-34c-5p (428). Interestingly, miR-34c, was also found upregulated in peripheral blood leukocytes of depression patients (429). The fact that this miRNA was also found dysregulated in peripheral leukocytes, increasing its potential as a reliable biomarker for inflammation-related depression. However, the contribution of miR-34c to immune cells activation (i.e., microglia and/or macrophages) and cytokine production needs to be further evaluated. A mice model of depression, induced by chronic unpredictable mild stress (CUMS), produced an increase in the expression of miR-134 and miR-124 and downregulation of miR-132 in the hippocampus (430). Indeed, miR-124 was also found upregulated in the PFC of depressed rats and in PBMCs of depression patients (431, 432). Its upregulation in depression has been associated with a physiological mechanism of resilience and adaptation to stress and depression behaviours, rather than a pathological role. In turn, miR-134 and miR-132 levels detected in the plasma of depression patients contradict the expression patterns detected in the brain (433, 434). In line with that, miR-1202, a primate-specific and brain-enriched miRNA, was reported to be downregulated in the PFC of depression patients (435). Nonetheless, miR-1202 levels were later evaluated in peripheral blood of depression patients, and despite being found downregulated in a first cohort of patients, these differences disappeared in a second larger cohort of patients (436). Also, miR-16, found upregulated in the hippocampus of early life stress-induced depressive rats, was later reported to be downregulated in the serum of depression patients (437, 438). In fact, most of the studies find major drawbacks in the reproducibility in different cohorts and

in the translation of the same brain expression patterns to the circulatory system, where the diagnostic is preferable. Although there may be multiple explanations for these differences, including biological reasons, it seems that a major need in the area is to complement observational studies with a deeper understanding of the mechanisms action of each miRNA candidate. Particularly, the identification of dysregulated miRNAs in body fluids of depression patients' must be followed by functional studies clarifying their role in disease, in order to fully elucidate their potential as specific and reliable biomarkers. For instance, in the context of inflammation-related depression, understanding how these dysregulated miRNAs influence microglia activation, and how they mediate the interplay between inflammation and the HPA axis, would provide increased knowledge about their role on specific disease-associated pathways.

3. Conclusion

Depression is one of the leading causes of disability worldwide, with one third of the patients not responding to treatments (439). Increasing evidences demonstrate that inflammation is intimately related with depressive symptoms in a bidirectional association. First, certain patients diagnosed with depression exhibit increased systemic levels of inflammatory biomarkers while, at the same time, chronic inflammatory diseases serve as a risk factor for the future development of depression. Pro-inflammatory cytokines are pointed as a main link between systemic inflammation and the CNS, since they can access the brain and activate local inflammatory networks to produce neurotransmitter function alterations. Furthermore, animal models of exogenous cytokine administration promote behavioural alterations including cytokine-induced depression and have been used to better understand the mechanisms by which cytokines affect neurotransmitter and neuroendocrine systems involved on neuropsychiatric illness. Therefore, the development of treatment strategies for depression that target inflammatory molecules, particularly in chronic inflammatory conditions, is already being the object of clinical trials and will likely continue to be a hot topic in the future. Obvious targets include CNS immune cells (e.g., microglia and infiltrating monocytes/macrophages), cytokines themselves, their signaling pathways, or other up- and downstream inflammatory mediators. In this context, miRNAs have received increasing interest due to their capacity to simultaneously regulate distinct relevant disease-associated pathways. miRNAs control the expression of multiple protein targets and have been implicated in several mechanisms of neural plasticity, neurogenesis, stress, and neuroinflammation, providing strong evidence that they may not only play critical roles in depression pathogenesis but are also potential therapeutic tools. The identification and clarification of the mechanism of action of depression-associated miRNAs will provide

insights for a better understanding of the diverse clinical entities, commonly known as depression, and their underlying biological pathways. This will contribute to provide more personalized and effective treatments, targeting specific forms of depression. Currently, prescription of the same classes of conventional antidepressants for all depression patients is far from having the desired results. Thus, it remains important to 1) understand the involvement of miRNAs in microglia activation, 2) investigate the behavioural and neuroinflammatory consequences of microglia activation and miRNA dysregulation, and to 3) consolidate these discoveries with the interplay between peripheral inflammatory markers and miRNAs candidates. This will provide sustained knowledge on the potential of specific miRNAs to be used as reliable biomarkers, improving depression diagnosis, and allowing the monitoring of patient's response to treatment.

References

1. Organization WH. Depression 2020 [updated 30 January 2020. Available from: http://www.who.int/mediacentre/factsheets/fs369/en/.
2. James SL, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet. 2018;392(10159):1789-858.
3. Vos T, Lim SS, Abbafati C, Abbas KM, Abbasi M, Abbasifard M, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. The Lancet. 2020;396(10258):1204-22.
4. Sixty-sixth World Health Assembly: WHA66.8 - Comprehensive mental health action plan 2013–2020 Geneva: World Health Organization; 2013.
5. Gold PW, Machado-Vieira R, Pavlatou MG. Clinical and biochemical manifestations of depression: relation to the neurobiology of stress. Neural plasticity. 2015;2015:581976.
6. Fiore V, Marci M, Poggi A, Giagulli VA, Licchelli B, Iacoviello M, et al. The association between diabetes and depression: a very disabling condition. Endocrine. 2015;48(1):14-24.
7. Wu Y, Sun D, Wang B, Li Y, Ma Y. The relationship of depressive symptoms and functional and structural markers of subclinical atherosclerosis: A systematic review and meta-analysis. European journal of preventive cardiology. 2018;25(7):706-16.
8. Frank E, Thase ME. Natural history and preventative treatment of recurrent mood disorders. Annual review of medicine. 1999;50:453-68.
9. Global Health Estimates 2015: Deaths by cause, age, sex, by country and by region, 2000–2015. Geneva: World Health Organization; 2016.
10. Depressive Disorders. Diagnostic and Statistical Manual of Mental Disorders. DSM Library: American Psychiatric Association; 2013.
11. Smith KM, Renshaw PF, Bilello J. The diagnosis of depression: current and emerging methods. Compr Psychiatry. 2013;54(1):1-6.
12. Fried EI, Nesse RM. Depression sum-scores don't add up: why analyzing specific depression symptoms is essential. BMC medicine. 2015;13(1):72.
13. Health Quality O. Psychotherapy for Major Depressive Disorder and Generalized Anxiety Disorder: A Health Technology Assessment. Ont Health Technol Assess Ser. 2017;17(15):1-167.
14. Cuijpers P, Quero S, Noma H, Ciharova M, Miguel C, Karyotaki E, et al. Psychotherapies for depression: a network meta-analysis covering efficacy, acceptability and long-term outcomes of all main treatment types. World Psychiatry. 2021;20(2):283-93.

15. Taylor C, Fricker AD, Devi LA, Gomes I. Mechanisms of action of antidepressants: from neurotransmitter systems to signaling pathways. Cellular Signalling. 2005;17(5):549-57.
16. Pigott HE. The STAR*D Trial: It Is Time to Reexamine the Clinical Beliefs That Guide the Treatment of Major Depression. Canadian Journal of Psychiatry Revue Canadienne de Psychiatrie. 2015;60(1):9-13.
17. Abbott R, Whear R, Nikolaou V, Bethel A, Coon JT, Stein K, et al. Tumour necrosis factor-alpha inhibitor therapy in chronic physical illness: A systematic review and meta-analysis of the effect on depression and anxiety. Journal of psychosomatic research. 2015;79(3):175-84.
18. O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. Journal of psychiatric research. 2007;41(3-4):326-31.
19. Fitzgerald P, O'Brien SM, Scully P, Rijkers K, Scott LV, Dinan TG. Cutaneous glucocorticoid receptor sensitivity and pro-inflammatory cytokine levels in antidepressant-resistant depression. Psychological medicine. 2006;36(1):37-43.
20. Harrison NA, Brydon L, Walker C, Gray MA, Steptoe A, Critchley HD. Inflammation causes mood changes through alterations in subgenual cingulate activity and mesolimbic connectivity. Biological psychiatry. 2009;66(5):407-14.
21. Lanquillon S, Krieg JC, Bening-Abu-Shach U, Vedder H. Cytokine production and treatment response in major depressive disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2000;22(4):370-9.
22. Sluzewska A, Sobieska M, Rybakowski JK. Changes in acute-phase proteins during lithium potentiation of antidepressants in refractory depression. Neuropsychobiology. 1997;35(3):123-7.
23. Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S, et al. Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Archives of general psychiatry. 1994;51(1):8-19.
24. Dantzer R, Kelley KW. Stress and immunity: an integrated view of relationships between the brain and the immune system. Life Sci. 1989;44(26):1995-2008.
25. Hart BL. Biological basis of the behavior of sick animals. Neuroscience and biobehavioral reviews. 1988;12(2):123-37.
26. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience. 2008;9(1):46-56.

27. Maier SF, Watkins LR. Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. Psychol Rev. 1998;105(1):83-107.
28. Smith RS. The macrophage theory of depression. Med Hypotheses. 1991;35(4):298-306.
29. Maes M, Bosmans E, Suy E, Vandervorst C, DeJonckheere C, Raus J. Depression-related disturbances in mitogen-induced lymphocyte responses and interleukin-1 beta and soluble interleukin-2 receptor production. Acta psychiatrica Scandinavica. 1991;84(4):379-86.
30. Maes M, Lambrechts J, Bosmans E, Jacobs J, Suy E, Vandervorst C, et al. Evidence for a systemic immune activation during depression: results of leukocyte enumeration by flow cytometry in conjunction with monoclonal antibody staining. Psychological medicine. 1992;22(1):45-53.
31. Maes M, Scharpe S, Bosmans E, Vandewoude M, Suy E, Uyttenbroeck W, et al. Disturbances in acute phase plasma proteins during melancholia: additional evidence for the presence of an inflammatory process during that illness. Progress in neuro-psychopharmacology & biological psychiatry. 1992;16(4):501-15.
32. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat Rev Immunol. 2014;14(6):392-404.
33. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 2005;5(12):953-64.
34. Reader BF, Jarrett BL, McKim DB, Wohleb ES, Godbout JP, Sheridan JF. Peripheral and central effects of repeated social defeat stress: monocyte trafficking, microglial activation, and anxiety. Neuroscience. 2015;289:429-42.
35. Torres-Platas SG, Cruceanu C, Chen GG, Turecki G, Mechawar N. Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides. Brain, behavior, and immunity. 2014;42:50-9.
36. Denicoff KD, Rubinow DR, Papa MZ, Simpson C, Seipp CA, Lotze MT, et al. The neuropsychiatric effects of treatment with interleukin-2 and lymphokine-activated killer cells. Annals of internal medicine. 1987;107(3):293-300.
37. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol. 2006;27(1):24-31.
38. Capuron L, Ravaud A, Dantzer R. Early depressive symptoms in cancer patients receiving interleukin 2 and/or interferon alfa-2b therapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2000;18(10):2143-51.
39. Constant A, Castera L, Dantzer R, Couzigou P, de Ledinghen V, Demotes-Mainard J, et al. Mood alterations during interferon-alfa therapy in patients with chronic hepatitis C:

evidence for an overlap between manic/hypomanic and depressive symptoms. The Journal of clinical psychiatry. 2005;66(8):1050-7.
40. Kim S, Foley FW, Picone MA, Halper J, Zemon V. Depression Levels and Interferon Treatment in People with Multiple Sclerosis. International Journal of MS Care. 2012;14(1):10-6.
41. Chiu WC, Su YP, Su KP, Chen PC. Recurrence of depressive disorders after interferon-induced depression. Translational psychiatry. 2017;7(2):e1026.
42. Udina M, Castellvi P, Moreno-Espana J, Navines R, Valdes M, Forns X, et al. Interferon-induced depression in chronic hepatitis C: a systematic review and meta-analysis. The Journal of clinical psychiatry. 2012;73(8):1128-38.
43. Murakami Y, Ishibashi T, Tomita E, Imamura Y, Tashiro T, Watcharanurak K, et al. Depressive symptoms as a side effect of Interferon-α therapy induced by induction of indoleamine 2,3-dioxygenase 1. Scientific Reports. 2016;6:29920.
44. Raison CL, Demetrashvili M, Capuron L, Miller AH. Neuropsychiatric adverse effects of interferon-alpha: recognition and management. CNS drugs. 2005;19(2):105-23.
45. Valentine AD, Meyers CA. Successful treatment of interferon-alpha-induced mood disorder with nortriptyline. Psychosomatics. 1995;36(4):418-9.
46. Musselman DL, Lawson DH, Gumnick JF, Manatunga AK, Penna S, Goodkin RS, et al. Paroxetine for the prevention of depression induced by high-dose interferon alfa. The New England journal of medicine. 2001;344(13):961-6.
47. Kraus MR, Schäfer A, Schöttker K, Keicher C, Weissbrich B, Hofbauer I, et al. Therapy of interferon-induced depression in chronic hepatitis C with citalopram: a randomised, double-blind, placebo-controlled study. Gut. 2008;57(4):531.
48. Gupta RK, Kumar R, Bassett M. Interferon-induced depressive illness in hep C patients responds to SSRI antidepressant treatments. Neuropsychiatric disease and treatment. 2006;2(3):355-8.
49. Morikawa O, Sakai N, Obara H, Saito N. Effects of interferon-alpha, interferon-gamma and cAMP on the transcriptional regulation of the serotonin transporter. European journal of pharmacology. 1998;349(2-3):317-24.
50. Zhu CB, Blakely RD, Hewlett WA. The proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha activate serotonin transporters. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2006;31(10):2121-31.
51. Banks WA, Kastin AJ, Gutierrez EG. Penetration of interleukin-6 across the murine blood-brain barrier. Neurosci Lett. 1994;179(1-2):53-6.
52. Banks WA, Kastin AJ, Broadwell RD. Passage of cytokines across the blood-brain barrier. Neuroimmunomodulation. 1995;2(4):241-8.

53. Yirmiya R, Rimmerman N, Reshef R. Depression as a microglial disease. Trends in neurosciences. 2015;38(10):637-58.
54. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science (New York, NY). 2005;308.
55. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P. Synaptic pruning by microglia is necessary for normal brain development. Science (New York, NY). 2011;333.
56. Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, et al. The classical complement cascade mediates CNS synapse elimination. Cell. 2007;131(6):1164-78.
57. Limatola C, Ransohoff RM. Modulating neurotoxicity through CX3CL1/CX3CR1 signaling. Frontiers in Cellular Neuroscience. 2014;8(229).
58. Meeuwsen S, Persoon-Deen C, Bsibsi M, Ravid R, van Noort JM. Cytokine, chemokine and growth factor gene profiling of cultured human astrocytes after exposure to proinflammatory stimuli. Glia. 2003;43(3):243-53.
59. Pang Y, Cai Z, Rhodes PG. Analysis of genes differentially expressed in astrocytes stimulated with lipopolysaccharide using cDNA arrays. Brain Res. 2001;914(1-2):15-22.
60. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci. 2011;14(9):1142-9.
61. Yamasaki R, Lu H, Butovsky O, Ohno N, Rietsch AM, Cialic R, et al. Differential roles of microglia and monocytes in the inflamed central nervous system. The Journal of Experimental Medicine. 2014;211(8):1533-49.
62. Sawicki CM, McKim DB, Wohleb ES, Jarrett BL, Reader BF, Norden DM, et al. Social defeat promotes a reactive endothelium in a brain region-dependent manner with increased expression of key adhesion molecules, selectins and chemokines associated with the recruitment of myeloid cells to the brain. Neuroscience. 2015;302:151-64.
63. Tong L, Gong Y, Wang P, Hu W, Wang J, Chen Z, et al. Microglia Loss Contributes to the Development of Major Depression Induced by Different Types of Chronic Stresses. Neurochemical research. 2017;42(10):2698-711.
64. Grigoleit JS, Kullmann JS, Wolf OT, Hammes F, Wegner A, Jablonowski S, et al. Dose-dependent effects of endotoxin on neurobehavioral functions in humans. PloS one. 2011;6(12):e28330.
65. Reichenberg A, Yirmiya R, Schuld A, Kraus T, Haack M, Morag A, et al. Cytokine-associated emotional and cognitive disturbances in humans. Archives of general psychiatry. 2001;58(5):445-52.

66. Frank MG, Baratta MV, Sprunger DB, Watkins LR, Maier SF. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. Brain, behavior, and immunity. 2007;21(1):47-59.
67. Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, et al. beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011;31(17):6277-88.
68. Frank MG, Miguel ZD, Watkins LR, Maier SF. Prior exposure to glucocorticoids sensitizes the neuroinflammatory and peripheral inflammatory responses to E. coli lipopolysaccharide. Brain, behavior, and immunity. 2010;24(1):19-30.
69. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nat Rev Immunol. 2016;16(1):22-34.
70. Leventopoulos M, Ruedi-Bettschen D, Knuesel I, Feldon J, Pryce CR, Opacka-Juffry J. Long-term effects of early life deprivation on brain glia in Fischer rats. Brain Res. 2007;1142:119-26.
71. Nagy C, Suderman M, Yang J, Szyf M, Mechawar N, Ernst C, et al. Astrocytic abnormalities and global DNA methylation patterns in depression and suicide. Molecular psychiatry. 2014;20:320.
72. Tynan RJ, Beynon SB, Hinwood M, Johnson SJ, Nilsson M, Woods JJ, et al. Chronic stress-induced disruption of the astrocyte network is driven by structural atrophy and not loss of astrocytes. Acta Neuropathol. 2013;126(1):75-91.
73. Hodes GE, Kana V, Menard C, Merad M, Russo SJ. Neuroimmune mechanisms of depression. Nat Neurosci. 2015;18(10):1386-93.
74. Irwin MR, Miller AH. Depressive disorders and immunity: 20 years of progress and discovery. Brain, behavior, and immunity. 2007;21(4):374-83.
75. Burke HM, Davis MC, Otte C, Mohr DC. Depression and cortisol responses to psychological stress: a meta-analysis. Psychoneuroendocrinology. 2005;30(9):846-56.
76. Amsterdam A, Tajima K, Sasson R. Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. Biochemical pharmacology. 2002;64(5-6):843-50.
77. Marino F, Cosentino M. Adrenergic modulation of immune cells: an update. Amino acids. 2013;45(1):55-71.
78. Tracey KJ. The inflammatory reflex. Nature. 2002;420(6917):853-9.
79. Cohen S, Janicki-Deverts D, Doyle WJ, Miller GE, Frank E, Rabin BS, et al. Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. Proceedings of the National Academy of Sciences. 2012;109(16):5995-9.

80. Miller AH. Inflammation Versus Glucocorticoids as Purveyors of Pathology during Stress: Have We Reached the Tipping Point? Biological psychiatry. 2008;64(4):263-5.
81. Avitsur R, Powell N, Padgett DA, Sheridan JF. Social interactions, stress, and immunity. Immunology and allergy clinics of North America. 2009;29(2):285-93.
82. Engler H, Bailey MT, Engler A, Stiner-Jones LM, Quan N, Sheridan JF. Interleukin-1 receptor type 1-deficient mice fail to develop social stress-associated glucocorticoid resistance in the spleen. Psychoneuroendocrinology. 2008;33(1):108-17.
83. Manz MG, Boettcher S. Emergency granulopoiesis. Nat Rev Immunol. 2014;14(5):302-14.
84. Pariante CM. The glucocorticoid receptor: part of the solution or part of the problem? Journal of psychopharmacology (Oxford, England). 2006;20(4 Suppl):79-84.
85. Horowitz MA, Zunszain PA. Neuroimmune and neuroendocrine abnormalities in depression: two sides of the same coin. Annals of the New York Academy of Sciences. 2015;1351:68-79.
86. Oxenkrug GF. Tryptophan–Kynurenine Metabolism as a Common Mediator of Genetic and Environmental Impacts in Major Depressive Disorder: The Serotonin Hypothesis Revisited 40 Years Later. The Israel journal of psychiatry and related sciences. 2010;47(1):56-63.
87. Felger JC, Lotrich FE. Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. Neuroscience. 2013;246:199-229.
88. Walter Magnus W. Monoamine reuptake inhibitors: highlights of recent research developments. Drug Development Research. 2005;65(3):97-118.
89. Heyes MP, Achim CL, Wiley CA, Major EO, Saito K, Markey SP. Human microglia convert l-tryptophan into the neurotoxin quinolinic acid. Biochemical Journal. 1996;320(Pt 2):595-7.
90. Steiner J, Walter M, Gos T, Guillemin GJ, Bernstein HG, Sarnyai Z, et al. Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission? Journal of neuroinflammation. 2011;8:94.
91. Dang Y, Dale WE, Brown OR. Comparative effects of oxygen on indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase of the kynurenine pathway. Free radical biology & medicine. 2000;28(4):615-24.
92. Fukui S, Schwarcz R, Rapoport SI, Takada Y, Smith QR. Blood-brain barrier transport of kynurenines: implications for brain synthesis and metabolism. J Neurochem. 1991;56(6):2007-17.

93. Okuda S, Nishiyama N, Saito H, Katsuki H. 3-Hydroxykynurenine, an endogenous oxidative stress generator, causes neuronal cell death with apoptotic features and region selectivity. J Neurochem. 1998;70(1):299-307.
94. Braidy N, Grant R, Adams S, Guillemin GJ. Neuroprotective effects of naturally occurring polyphenols on quinolinic acid-induced excitotoxicity in human neurons. The FEBS journal. 2010;277(2):368-82.
95. Myint AM, Kim YK. Cytokine-serotonin interaction through IDO: a neurodegeneration hypothesis of depression. Medical hypotheses. 2003;61(5-6):519-25.
96. Myint AM, Kim YK, Verkerk R, Scharpe S, Steinbusch H, Leonard B. Kynurenine pathway in major depression: evidence of impaired neuroprotection. Journal of affective disorders. 2007;98(1-2):143-51.
97. Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, et al. T cell apoptosis by tryptophan catabolism. Cell death and differentiation. 2002;9(10):1069-77.
98. Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM, et al. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. Science (New York, NY). 2005;310(5749):850-5.
99. Schiepers OJ, Wichers MC, Maes M. Cytokines and major depression. Progress in neuro-psychopharmacology & biological psychiatry. 2005;29(2):201-17.
100. Owe-Young R, Webster NL, Mukhtar M, Pomerantz RJ, Smythe G, Walker D, et al. Kynurenine pathway metabolism in human blood-brain-barrier cells: implications for immune tolerance and neurotoxicity. J Neurochem. 2008;105(4):1346-57.
101. Carpenter LL, Anderson GM, Pelton GH, Gudin JA, Kirwin PD, Price LH, et al. Tryptophan depletion during continuous CSF sampling in healthy human subjects. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 1998;19(1):26-35.
102. Culley WJ, Saunders RN, Mertz ET, Jolly DH. Effect of a tryptophan deficient diet on brain serotonin and plasma tryptophan level. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY). 1963;113:645-8.
103. Delgado PL, Charney DS, Price LH, Aghajanian GK, Landis H, Heninger GR. Serotonin function and the mechanism of antidepressant action. Reversal of antidepressant-induced remission by rapid depletion of plasma tryptophan. Archives of general psychiatry. 1990;47(5):411-8.
104. Tilleux S, Hermans E. Neuroinflammation and regulation of glial glutamate uptake in neurological disorders. J Neurosci Res. 2007;85(10):2059-70.

105. Sanacora G, Treccani G, Popoli M. Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders. Neuropharmacology. 2012;62(1):63-77.
106. Zunszain PA, Anacker C, Cattaneo A, Carvalho LA, Pariante CM. Glucocorticoids, cytokines and brain abnormalities in depression. Progress in neuro-psychopharmacology & biological psychiatry. 2011;35(3):722-9.
107. Chapman BP, Moynihan J. The brain-skin connection: role of psychosocial factors and neuropeptides in psoriasis. Expert review of clinical immunology. 2009;5(6):623-7.
108. Moynihan J, Rieder E, Tausk F. Psychoneuroimmunology: the example of psoriasis. Giornale italiano di dermatologia e venereologia : organo ufficiale, Societa italiana di dermatologia e sifilografia. 2010;145(2):221-8.
109. Hughes JE, Barraclough BM, Hamblin LG, White JE. Psychiatric symptoms in dermatology patients. The British journal of psychiatry : the journal of mental science. 1983;143:51-4.
110. Fortune DG, Richards HL, Griffiths CE. Psychologic factors in psoriasis: consequences, mechanisms, and interventions. Dermatologic clinics. 2005;23(4):681-94.
111. Naldi L, Peli L, Parazzini F, Carrel CF. Family history of psoriasis, stressful life events, and recent infectious disease are risk factors for a first episode of acute guttate psoriasis: results of a case-control study. Journal of the American Academy of Dermatology. 2001;44(3):433-8.
112. Fortune DG, Richards HL, Griffiths CE, Main CJ. Psychological stress, distress and disability in patients with psoriasis: consensus and variation in the contribution of illness perceptions, coping and alexithymia. The British journal of clinical psychology. 2002;41(Pt 2):157-74.
113. Fortune DG, Richards HL, Griffiths CE, Main CJ. Targeting cognitive-behaviour therapy to patients' implicit model of psoriasis: results from a patient preference controlled trial. The British journal of clinical psychology. 2004;43(Pt 1):65-82.
114. Zachariae R, Oster H, Bjerring P, Kragballe K. Effects of psychologic intervention on psoriasis: a preliminary report. Journal of the American Academy of Dermatology. 1996;34(6):1008-15.
115. Kabat-Zinn J, Wheeler E, Light T, Skillings A, Scharf MJ, Cropley TG, et al. Influence of a mindfulness meditation-based stress reduction intervention on rates of skin clearing in patients with moderate to severe psoriasis undergoing phototherapy (UVB) and photochemotherapy (PUVA). Psychosomatic medicine. 1998;60(5):625-32.
116. Fleming P, Roubille C, Richer V, Starnino T, McCourt C, McFarlane A, et al. Effect of biologics on depressive symptoms in patients with psoriasis: a systematic review. Journal of the European Academy of Dermatology and Venereology : JEADV. 2015;29(6):1063-70.

117. Menter A, Augustin M, Signorovitch J, Yu AP, Wu EQ, Gupta SR, et al. The effect of adalimumab on reducing depression symptoms in patients with moderate to severe psoriasis: a randomized clinical trial. Journal of the American Academy of Dermatology. 2010;62(5):812-8.
118. de Mattos BR, Garcia MP, Nogueira JB, Paiatto LN, Albuquerque CG, Souza CL, et al. Inflammatory Bowel Disease: An Overview of Immune Mechanisms and Biological Treatments. Mediators of inflammation. 2015;2015:493012.
119. Bonaz B. Inflammatory bowel diseases: a dysfunction of brain-gut interactions? Minerva gastroenterologica e dietologica. 2013;59(3):241-59.
120. Bonaz BL, Bernstein CN. Brain-gut interactions in inflammatory bowel disease. Gastroenterology. 2013;144(1):36-49.
121. Ananthakrishnan AN, Khalili H, Pan A, Higuchi LM, de Silva P, Richter JM, et al. Association between depressive symptoms and incidence of Crohn's disease and ulcerative colitis: results from the Nurses' Health Study. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2013;11(1):57-62.
122. Goodhand JR, Greig FI, Koodun Y, McDermott A, Wahed M, Langmead L, et al. Do antidepressants influence the disease course in inflammatory bowel disease? A retrospective case-matched observational study. Inflammatory bowel diseases. 2012;18(7):1232-9.
123. Cawthorpe D, Davidson M. Temporal Comorbidity of Mental Disorder and Ulcerative Colitis. The Permanente Journal. 2015;19(1):52-7.
124. Vlachos II, Barbatis C, Tsopanomichalou M, Abou-Assabeh L, Goumas K, Ginieri-Coccossis M, et al. Correlation between depression, anxiety, and polymorphonuclear cells' resilience in ulcerative colitis: the mediating role of heat shock protein 70. BMC Gastroenterology. 2014;14:77-.
125. Mardini HE, Kip KE, Wilson JW. Crohn's disease: a two-year prospective study of the association between psychological distress and disease activity. Digestive diseases and sciences. 2004;49(3):492-7.
126. Mittermaier C, Dejaco C, Waldhoer T, Oefferlbauer-Ernst A, Miehsler W, Beier M, et al. Impact of depressive mood on relapse in patients with inflammatory bowel disease: a prospective 18-month follow-up study. Psychosomatic medicine. 2004;66(1):79-84.
127. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. The New England journal of medicine. 2011;365(23):2205-19.
128. Waraich P, Goldner EM, Somers JM, Hsu L. Prevalence and incidence studies of mood disorders: a systematic review of the literature. Can J Psychiatry. 2004;49(2):124-38.

129. Ang DC, Choi H, Kroenke K, Wolfe F. Comorbid depression is an independent risk factor for mortality in patients with rheumatoid arthritis. The Journal of rheumatology. 2005;32(6):1013-9.
130. van Hoogmoed D, Fransen J, Bleijenberg G, van Riel P. Physical and psychosocial correlates of severe fatigue in rheumatoid arthritis. Rheumatology (Oxford, England). 2010;49(7):1294-302.
131. Mikuls T, Saag K, Criswell L, Merlino L, Cerhan JR. Health related quality of life in women with elderly onset rheumatoid arthritis. The Journal of rheumatology. 2003;30(5):952-7.
132. Wolfe F, Michaud K. Predicting depression in rheumatoid arthritis: the signal importance of pain extent and fatigue, and comorbidity. Arthritis and rheumatism. 2009;61(5):667-73.
133. Vallerand IA, Lewinson RT, Frolkis AD, Lowerison MW, Kaplan GG, Swain MG, et al. Depression as a risk factor for the development of rheumatoid arthritis: a population-based cohort study. RMD Open. 2018;4(2).
134. Braley TJ, Chervin RD, Segal BM. Fatigue, Tiredness, Lack of Energy, and Sleepiness in Multiple Sclerosis Patients Referred for Clinical Polysomnography. Multiple Sclerosis International. 2012;2012:673936.
135. Patten SB, Beck CA, Williams JV, Barbui C, Metz LM. Major depression in multiple sclerosis: a population-based perspective. Neurology. 2003;61(11):1524-7.
136. Tarrants M, Oleen-Burkey M, Castelli-Haley J, Lage MJ. The impact of comorbid depression on adherence to therapy for multiple sclerosis. Mult Scler Int. 2011;2011:271321.
137. Patten SB, Williams JV, Lavorato DH, Koch M, Metz LM. Depression as a predictor of occupational transition in a multiple sclerosis cohort. Functional neurology. 2013;28(4):275-80.
138. Feinstein A. Multiple sclerosis, depression, and suicide. Clinicians should pay more attention to psychopathology. 1997;315(7110):691-2.
139. Feinstein A. An examination of suicidal intent in patients with multiple sclerosis. Neurology. 2002;59(5):674-8.
140. Pompili M, Forte A, Palermo M, Stefani H, Lamis DA, Serafini G, et al. Suicide risk in multiple sclerosis: a systematic review of current literature. Journal of psychosomatic research. 2012;73(6):411-7.
141. Raison CL, Rutherford RE, Woolwine BJ, et al. A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: The role of baseline inflammatory biomarkers. JAMA Psychiatry. 2013;70(1):31-41.
142. Langley RG, Feldman SR, Han C, Schenkel B, Szapary P, Hsu MC, et al. Ustekinumab significantly improves symptoms of anxiety, depression, and skin-related quality of life in

patients with moderate-to-severe psoriasis: Results from a randomized, double-blind, placebo-controlled phase III trial. Journal of the American Academy of Dermatology. 2010;63(3):457-65.
143. Brietzke E, Scheinberg M, Lafer B. Therapeutic potential of interleukin-6 antagonism in bipolar disorder. Medical hypotheses. 2011;76(1):21-3.
144. Iyengar RL, Gandhi S, Aneja A, Thorpe K, Razzouk L, Greenberg J, et al. NSAIDs are associated with lower depression scores in patients with osteoarthritis. The American journal of medicine. 2013;126(11):1017.e11-8.
145. Kohler O, Benros ME, Nordentoft M, Farkouh ME, Iyengar RL, Mors O, et al. Effect of anti-inflammatory treatment on depression, depressive symptoms, and adverse effects: a systematic review and meta-analysis of randomized clinical trials. JAMA Psychiatry. 2014;71(12):1381-91.
146. Warner-Schmidt JL, Vanover KE, Chen EY, Marshall JJ, Greengard P. Antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) are attenuated by antiinflammatory drugs in mice and humans. Proc Natl Acad Sci U S A. 2011;108(22):9262-7.
147. Fourrier C, Sampson E, Mills NT, Baune BT. Anti-inflammatory treatment of depression: study protocol for a randomised controlled trial of vortioxetine augmented with celecoxib or placebo. Trials. 2018;19(1):447.
148. Mills NT, Sampson E, Fourrier C, Baune BT. Clinical Switching Strategies of Various Antidepressants to Vortioxetine in the PREDDICT Trial. International Journal of Neuropsychopharmacology. 2021;24(4):314-21.
149. Sluzewska A, Rybakowski JK, Laciak M, Mackiewicz A, Sobieska M, Wiktorowicz K. Interleukin-6 serum levels in depressed patients before and after treatment with fluoxetine. Annals of the New York Academy of Sciences. 1995;762:474-6.
150. Jazayeri S, Keshavarz SA, Tehrani-Doost M, Djalali M, Hosseini M, Amini H, et al. Effects of eicosapentaenoic acid and fluoxetine on plasma cortisol, serum interleukin-1beta and interleukin-6 concentrations in patients with major depressive disorder. Psychiatry research. 2010;178(1):112-5.
151. Kubera M, Kenis G, Bosmans E, Kajta M, Basta-Kaim A, Scharpe S, et al. Stimulatory effect of antidepressants on the production of IL-6. International immunopharmacology. 2004;4(2):185-92.
152. Murrough JW, Iosifescu DV, Chang LC, Al Jurdi RK, Green CE, Perez AM, et al. Antidepressant efficacy of ketamine in treatment-resistant major depression: a two-site randomized controlled trial. The American journal of psychiatry. 2013;170(10):1134-42.
153. De Kock M, Loix S, Lavand'homme P. Ketamine and peripheral inflammation. CNS neuroscience & therapeutics. 2013;19(6):403-10.

154. Miller AH, Haroon E, Raison CL, Felger JC. Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. Depression and anxiety. 2013;30(4):297-306.
155. Miller AH, Maletic V, Raison CL. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biological psychiatry. 2009;65(9):732-41.
156. Raison CL, Felger JC, Miller AH. Inflammation and treatment resistance in major depression: The perfect storm. Psychiatric Times. 2013;30(9).
157. Eller T, Vasar V, Shlik J, Maron E. Pro-inflammatory cytokines and treatment response to escitaloprsam in major depressive disorder. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2008;32(2):445-50.
158. Lotrich FE. Inflammatory cytokine-associated depression. Brain Res. 2015;1617:113-25.
159. Strawbridge R, Young AH, Cleare AJ. Biomarkers for depression: recent insights, current challenges and future prospects. Neuropsychiatric disease and treatment. 2017;13:1245-62.
160. Postal M, Lapa AT, Sinicato NA, de Oliveira Peliçari K, Peres FA, Costallat LTL, et al. Depressive symptoms are associated with tumor necrosis factor alpha in systemic lupus erythematosus. Journal of neuroinflammation. 2016;13:5.
161. Farooq RK, Asghar K, Kanwal S, Zulqernain A. Role of inflammatory cytokines in depression: Focus on interleukin-1beta. Biomedical reports. 2017;6(1):15-20.
162. Howren MB, Lamkin DM, Suls J. Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. Psychosomatic medicine. 2009;71(2):171-86.
163. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A meta-analysis of cytokines in major depression. Biological psychiatry. 2010;67(5):446-57.
164. Strawbridge R, Arnone D, Danese A, Papadopoulos A, Herane Vives A, Cleare AJ. Inflammation and clinical response to treatment in depression: A meta-analysis. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2015;25(10):1532-43.
165. Hannestad J, DellaGioia N, Bloch M. The effect of antidepressant medication treatment on serum levels of inflammatory cytokines: a meta-analysis. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2011;36(12):2452-9.
166. Quan N, Banks WA. Brain-immune communication pathways. Brain, behavior, and immunity. 2007;21(6):727-35.
167. Mantovani A, Bonecchi R, Locati M. Tuning inflammation and immunity by chemokine sequestration: decoys and more. Nature Reviews Immunology. 2006;6:907.
168. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. The New England journal of medicine. 2006;354(6):610-21.

169. Stuart MJ, Singhal G, Baune BT. Systematic Review of the Neurobiological Relevance of Chemokines to Psychiatric Disorders. Front Cell Neurosci. 2015;9:357.
170. Stuart MJ, Baune BT. Chemokines and chemokine receptors in mood disorders, schizophrenia, and cognitive impairment: a systematic review of biomarker studies. Neuroscience and biobehavioral reviews. 2014;42:93-115.
171. de Haas AH, van Weering HR, de Jong EK, Boddeke HW, Biber KP. Neuronal chemokines: versatile messengers in central nervous system cell interaction. Mol Neurobiol. 2007;36(2):137-51.
172. Gao Y-J, Ji R-R. Chemokines, neuronal-glial interactions, and central processing of neuropathic pain. Pharmacology & therapeutics. 2010;126(1):56-68.
173. Banisadr G, Gosselin RD, Mechighel P, Kitabgi P, Rostene W, Parsadaniantz SM. Highly regionalized neuronal expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) in rat brain: evidence for its colocalization with neurotransmitters and neuropeptides. The Journal of comparative neurology. 2005;489(3):275-92.
174. Zhou Y, Tang H, Liu J, Dong J, Xiong H. Chemokine CCL2 modulation of neuronal excitability and synaptic transmission in rat hippocampal slices. Journal of neurochemistry. 2011;116(3):406-14.
175. Marciniak E, Faivre E, Dutar P, Alves Pires C, Demeyer D, Caillierez R, et al. The Chemokine MIP-1alpha/CCL3 impairs mouse hippocampal synaptic transmission, plasticity and memory. Sci Rep. 2015;5:15862.
176. Callewaere C, Banisadr G, Rostene W, Parsadaniantz SM. Chemokines and chemokine receptors in the brain: implication in neuroendocrine regulation. Journal of molecular endocrinology. 2007;38(3):355-63.
177. Li M, Hale JS, Rich JN, Ransohoff RM, Lathia JD. Chemokine CXCL12 in neurodegenerative diseases: a S.O.S signal for stem cell-based repair. Trends in neurosciences. 2012;35(10):619-28.
178. Biber K, Vinet J, Boddeke HWGM. Neuron-microglia signaling: Chemokines as versatile messengers. Journal of Neuroimmunology. 2008;198(1):69-74.
179. Eyre HA, Air T, Pradhan A, Johnston J, Lavretsky H, Stuart MJ, et al. A meta-analysis of chemokines in major depression. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2016;68:1-8.
180. Kohler CA, Freitas TH, Maes M, de Andrade NQ, Liu CS, Fernandes BS, et al. Peripheral cytokine and chemokine alterations in depression: a meta-analysis of 82 studies. Acta psychiatrica Scandinavica. 2017;135(5):373-87.
181. Lehto SM, Niskanen L, Herzig KH, Tolmunen T, Huotari A, Viinamaki H, et al. Serum chemokine levels in major depressive disorder. Psychoneuroendocrinology. 2010;35(2):226-32.

182. Baune BT, Smith E, Reppermund S, Air T, Samaras K, Lux O, et al. Inflammatory biomarkers predict depressive, but not anxiety symptoms during aging: the prospective Sydney Memory and Aging Study. Psychoneuroendocrinology. 2012;37(9):1521-30.
183. Leighton SP, Nerurkar L, Krishnadas R, Johnman C, Graham GJ, Cavanagh J. Chemokines in depression in health and in inflammatory illness: a systematic review and meta-analysis. Molecular psychiatry. 2017;23:48.
184. Grassi-Oliveira R, Brieztke E, Teixeira A, Pezzi JC, Zanini M, Lopes RP, et al. Peripheral chemokine levels in women with recurrent major depression with suicidal ideation. Revista brasileira de psiquiatria (Sao Paulo, Brazil : 1999). 2012;34(1):71-5.
185. Merendino RA, Di Pasquale G, De Luca F, Di Pasquale L, Ferlazzo E, Martino G, et al. Involvement of fractalkine and macrophage inflammatory protein-1 alpha in moderate-severe depression. Mediators of inflammation. 2004;13(3):205-7.
186. Eller T, Vasar V, Shlik J, Maron E. Effects of bupropion augmentation on pro-inflammatory cytokines in escitalopram-resistant patients with major depressive disorder. Journal of psychopharmacology (Oxford, England). 2009;23(7):854-8.
187. Kruse JL, Congdon E, Olmstead R, Njau S, Breen EC, Narr KL, et al. Inflammation and Improvement of Depression Following Electroconvulsive Therapy in Treatment-Resistant Depression. The Journal of clinical psychiatry. 2018;79(2).
188. Kiraly DD, Horn SR, Van Dam NT, Costi S, Schwartz J, Kim-Schulze S, et al. Altered peripheral immune profiles in treatment-resistant depression: response to ketamine and prediction of treatment outcome. Translational psychiatry. 2017;7:e1065.
189. Eklund CM. Proinflammatory cytokines in CRP baseline regulation. Advances in clinical chemistry. 2009;48:111-36.
190. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. Journal of Clinical Investigation. 2003;111(12):1805-12.
191. Bassuk SS, Rifai N, Ridker PM. High-sensitivity C-reactive protein: clinical importance. Current problems in cardiology. 2004;29(8):439-93.
192. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO, 3rd, Criqui M, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 2003;107(3):499-511.
193. Wium-Andersen MK, Orsted DD, Nielsen SF, Nordestgaard BG. Elevated C-reactive protein levels, psychological distress, and depression in 73131 individuals. JAMA Psychiatry. 2013;70(2):176-84.
194. Liukkonen T, Silvennoinen-Kassinen S, Jokelainen J, Rasanen P, Leinonen M, Meyer-Rochow VB, et al. The association between C-reactive protein levels and depression: Results from the northern Finland 1966 birth cohort study. Biological psychiatry. 2006;60(8):825-30.

195. Danner M, Kasl SV, Abramson JL, Vaccarino V. Association between depression and elevated C-reactive protein. Psychosomatic medicine. 2003;65(3):347-56.
196. Elovainio M, Aalto AM, Kivimaki M, Pirkola S, Sundvall J, Lonnqvist J, et al. Depression and C-reactive protein: population-based Health 2000 Study. Psychosomatic medicine. 2009;71(4):423-30.
197. Bjerkeset O, Romild U, Smith GD, Hveem K. The associations of high levels of C-reactive protein with depression and myocardial infarction in 9258 women and men from the HUNT population study. Psychological medicine. 2011;41(2):345-52.
198. Raison CL, Pikalov A, Siu C, Tsai J, Koblan K, Loebel A. C-reactive protein and response to lurasidone in patients with bipolar depression. Brain, behavior, and immunity. 2018.
199. Cepeda MS, Stang P, Makadia R. Depression Is Associated With High Levels of C-Reactive Protein and Low Levels of Fractional Exhaled Nitric Oxide: Results From the 2007-2012 National Health and Nutrition Examination Surveys. The Journal of clinical psychiatry. 2016;77(12):1666-71.
200. Krishnan V, Nestler EJ. Animal Models of Depression: Molecular Perspectives. Current topics in behavioral neurosciences. 2011;7:121-47.
201. Murgatroyd C, Quinn JP, Sharp HM, Pickles A, Hill J. Effects of prenatal and postnatal depression, and maternal stroking, at the glucocorticoid receptor gene. Translational psychiatry. 2015;5(5):e560.
202. Fumagalli F, Bedogni F, Perez J, Racagni G, Riva MA. Corticostriatal brain-derived neurotrophic factor dysregulation in adult rats following prenatal stress. The European journal of neuroscience. 2004;20(5):1348-54.
203. Diz-Chaves Y, Pernia O, Carrero P, Garcia-Segura LM. Prenatal stress causes alterations in the morphology of microglia and the inflammatory response of the hippocampus of adult female mice. Journal of neuroinflammation. 2012;9:71.
204. Heim C, Binder EB. Current research trends in early life stress and depression: review of human studies on sensitive periods, gene-environment interactions, and epigenetics. Experimental neurology. 2012;233(1):102-11.
205. Negele A, Kaufhold J, Kallenbach L, Leuzinger-Bohleber M. Childhood Trauma and Its Relation to Chronic Depression in Adulthood. Depress Res Treat. 2015;2015:650804-.
206. Récamier-Carballo S, Estrada-Camarena E, López-Rubalcava C. Maternal separation induces long-term effects on monoamines and brain-derived neurotrophic factor levels on the frontal cortex, amygdala, and hippocampus: differential effects after a stress challenge. Behavioural pharmacology. 2017;28(7):545-57.

207. Dutcher EG, Pama EAC, Lynall M-E, Khan S, Clatworthy MR, Robbins TW, et al. Early-life stress and inflammation: A systematic review of a key experimental approach in rodents. Brain and Neuroscience Advances. 2020;4:2398212820978049.
208. Wieck A, Andersen SL, Brenhouse HC. Evidence for a neuroinflammatory mechanism in delayed effects of early life adversity in rats: Relationship to cortical NMDA receptor expression. Brain, behavior, and immunity. 2013;28:218-26.
209. Marquez C, Poirier GL, Cordero MI, Larsen MH, Groner A, Marquis J, et al. Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression. Translational psychiatry. 2013;3:e216.
210. Papilloud A, Guillot de Suduiraut I, Zanoletti O, Grosse J, Sandi C. Peripubertal stress increases play fighting at adolescence and modulates nucleus accumbens CB1 receptor expression and mitochondrial function in the amygdala. Translational psychiatry. 2018;8(1):156.
211. Brenes Saenz JC, Villagra OR, Fornaguera Trias J. Factor analysis of Forced Swimming test, Sucrose Preference test and Open Field test on enriched, social and isolated reared rats. Behavioural brain research. 2006;169(1):57-65.
212. Anisman H, Merali Z. Rodent models of depression: learned helplessness induced in mice. Current protocols in neuroscience. 2001;Chapter 8:Unit 8.10C.
213. Landgraf D, Long J, Der-Avakian A, Streets M, Welsh DK. Dissociation of Learned Helplessness and Fear Conditioning in Mice: A Mouse Model of Depression. PloS one. 2015;10(4):e0125892.
214. Willner P. The chronic mild stress (CMS) model of depression: History, evaluation and usage. Neurobiol Stress. 2017;6:78-93.
215. Nollet M, Le Guisquet AM, Belzung C. Models of depression: unpredictable chronic mild stress in mice. Current protocols in pharmacology. 2013;Chapter 5:Unit 5.65.
216. Stepanichev M, Dygalo NN, Grigoryan G, Shishkina GT, Gulyaeva N. Rodent models of depression: neurotrophic and neuroinflammatory biomarkers. BioMed research international. 2014;2014:932757.
217. Golden SA, Covington HE, 3rd, Berton O, Russo SJ. A standardized protocol for repeated social defeat stress in mice. Nature protocols. 2011;6(8):1183-91.
218. Reader BF, Jarrett BL, McKim DB, Wohleb ES, Godbout JP, Sheridan JF. Peripheral and Central Effects of Repeated Social Defeat Stress: Monocyte Trafficking, Microglial Activation, and Anxiety. Neuroscience. 2015;289:429-42.
219. Remus JL, Dantzer R. Inflammation Models of Depression in Rodents: Relevance to Psychotropic Drug Discovery. The international journal of neuropsychopharmacology. 2016;19(9).

220. Dantzer R. Cytokine, Sickness Behavior, and Depression. Neurologic Clinics. 2006;24(3):441-60.
221. O'Connor JC, Lawson MA, Andre C, Moreau M, Lestage J, Castanon N, et al. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Molecular psychiatry. 2009;14(5):511-22.
222. Laumet G, Edralin JD, Chiang AC-A, Dantzer R, Heijnen CJ, Kavelaars A. Resolution of inflammation-induced depression requires T lymphocytes and endogenous brain interleukin-10 signaling. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2018.
223. Goshen I, Kreisel T, Ben-Menachem-Zidon O, Licht T, Weidenfeld J, Ben-Hur T, et al. Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. Molecular psychiatry. 2008;13(7):717-28.
224. Koo JW, Russo SJ, Ferguson D, Nestler EJ, Duman RS. Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. Proc Natl Acad Sci U S A. 2010;107(6):2669-74.
225. Sukoff Rizzo SJ, Neal SJ, Hughes ZA, Beyna M, Rosenzweig-Lipson S, Moss SJ, et al. Evidence for sustained elevation of IL-6 in the CNS as a key contributor of depressive-like phenotypes. Translational psychiatry. 2012;2:e199.
226. Henry CJ, Huang Y, Wynne A, Hanke M, Himler J, Bailey MT, et al. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. Journal of neuroinflammation. 2008;5:15.
227. McKim DB, Niraula A, Tarr AJ, Wohleb ES, Sheridan JF, Godbout JP. Neuroinflammatory Dynamics Underlie Memory Impairments after Repeated Social Defeat. The Journal of Neuroscience. 2016;36(9):2590.
228. De La Garza R, 2nd, Asnis GM. The non-steroidal anti-inflammatory drug diclofenac sodium attenuates IFN-alpha induced alterations to monoamine turnover in prefrontal cortex and hippocampus. Brain Res. 2003;977(1):70-9.
229. Fischer CW, Eskelund A, Budac DP, Tillmann S, Liebenberg N, Elfving B, et al. Interferon-alpha treatment induces depression-like behaviour accompanied by elevated hippocampal quinolinic acid levels in rats. Behavioural brain research. 2015;293:166-72.
230. Karson A, Demirtas T, Bayramgurler D, Balci F, Utkan T. Chronic administration of infliximab (TNF-alpha inhibitor) decreases depression and anxiety-like behaviour in rat model of chronic mild stress. Basic & clinical pharmacology & toxicology. 2013;112(5):335-40.
231. Haapakoski R, Mathieu J, Ebmeier KP, Alenius H, Kivimaki M. Cumulative meta-analysis of interleukins 6 and 1beta, tumour necrosis factor alpha and C-reactive protein in patients with major depressive disorder. Brain, behavior, and immunity. 2015;49:206-15.

232. Mutlu O, Gumuslu E, Ulak G, Celikyurt IK, Kokturk S, Kır HM, et al. Effects of fluoxetine, tianeptine and olanzapine on unpredictable chronic mild stress-induced depression-like behavior in mice. Life Sciences. 2012;91(25):1252-62.
233. Hanke ML, Powell ND, Stiner LM, Bailey MT, Sheridan JF. Beta adrenergic blockade decreases the immunomodulatory effects of social disruption stress(). Brain, behavior, and immunity. 2012;26(7):1150-9.
234. Carboni L, Becchi S, Piubelli C, Mallei A, Giambelli R, Razzoli M, et al. Early-life stress and antidepressants modulate peripheral biomarkers in a gene–environment rat model of depression. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2010;34(6):1037-48.
235. Song C, Zhang XY, Manku M. Increased phospholipase A2 activity and inflammatory response but decreased nerve growth factor expression in the olfactory bulbectomized rat model of depression: effects of chronic ethyl-eicosapentaenoate treatment. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29(1):14-22.
236. Palazzo AF, Lee ES. Non-coding RNA: what is functional and what is junk? Front Genet. 2015;6(2).
237. Esteller M. Non-coding RNAs in human disease. Nature Reviews Genetics. 2011;12(12):861-74.
238. Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutation research. 2011;717(1-2):1-8.
239. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843-54.
240. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993;75(5):855-62.
241. Reichholf B, Herzog VA, Fasching N, Manzenreither RA, Sowemimo I, Ameres SL. Time-Resolved Small RNA Sequencing Unravels the Molecular Principles of MicroRNA Homeostasis. Molecular Cell. 2019;75(4):756-68.e7.
242. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 2014;42(Database issue):D68-73.
243. O'Connell RM, Rao DS, Baltimore D. microRNA regulation of inflammatory responses. Annu Rev Immunol. 2012;30:295-312.
244. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature. 2008;455(7209):64-71.
245. Ha M, Kim VN. Regulation of microRNA biogenesis. Nature Reviews Molecular Cell Biology. 2014;15(8):509-24.
246. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nature Cell Biology. 2009;11(3):228-34.

247. Stavast CJ, Erkeland SJ. The Non-Canonical Aspects of MicroRNAs: Many Roads to Gene Regulation. Cells. 2019;8(11):1465.
248. Cheloufi S, Dos Santos CO, Chong MMW, Hannon GJ. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. Nature. 2010;465(7298):584-9.
249. Cifuentes D, Xue H, Taylor DW, Patnode H, Mishima Y, Cheloufi S, et al. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. Science (New York, NY). 2010;328(5986):1694-8.
250. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: synthesis, mechanism, function, and recent clinical trials. Biochimica et biophysica acta. 2010;1803(11):1231-43.
251. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes & development. 2006;20(5):515-24.
252. Kehl T, Backes C, Kern F, Fehlmann T, Ludwig N, Meese E, et al. About miRNAs, miRNA seeds, target genes and target pathways. Oncotarget. 2017;8(63):107167-75.
253. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. Nucleic Acids Res. 2019;47(D1):D155-d62.
254. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews Genetics. 2008;9(2):102-14.
255. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Frontiers in endocrinology. 2018;9:402.
256. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proceedings of the National Academy of Sciences. 2011;108(12):5003-8.
257. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids--the mix of hormones and biomarkers. Nature reviews Clinical oncology. 2011;8(8):467-77.
258. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nature Cell Biology. 2011;13(4):423-33.
259. Condrat CE, Thompson DC, Barbu MG, Bugnar OL, Boboc A, Cretoiu D, et al. miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. Cells. 2020;9(2):276.
260. Xi Y, Nakajima G, Gavin E, Morris CG, Kudo K, Hayashi K, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA. 2007;13(10):1668-74.

261. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004;64(11):3753-6.
262. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. The New England journal of medicine. 2005;353(17):1793-801.
263. Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, et al. Detection and characterization of placental microRNAs in maternal plasma. Clinical chemistry. 2008;54(3):482-90.
264. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. British journal of haematology. 2008;141(5):672-5.
265. Reuter JA, Spacek D, Snyder MP. High-Throughput Sequencing Technologies. Molecular cell. 2015;58(4):586-97.
266. Ludwig N, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, et al. Distribution of miRNA expression across human tissues. Nucleic Acids Research. 2016;44(8):3865-77.
267. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. PNAS. 2008;105(30):10513-8.
268. Bonneau E, Neveu B, Kostantin E, Tsongalis GJ, De Guire V. How close are miRNAs from clinical practice? A perspective on the diagnostic and therapeutic market. EJIFCC. 2019;30(2):114-27.
269. Meiri E, Mueller WC, Rosenwald S, Zepeniuk M, Klinke E, Edmonston TB, et al. A second-generation microRNA-based assay for diagnosing tumor tissue origin. The oncologist. 2012;17(6):801-12.
270. Labourier E, Shifrin A, Busseniers AE, Lupo MA, Manganelli ML, Andruss B, et al. Molecular Testing for miRNA, mRNA, and DNA on Fine-Needle Aspiration Improves the Preoperative Diagnosis of Thyroid Nodules With Indeterminate Cytology. The Journal of clinical endocrinology and metabolism. 2015;100(7):2743-50.
271. Heilmeier U, Hackl M, Skalicky S, Weilner S, Schroeder F, Vierlinger K, et al. Serum miRNA Signatures Are Indicative of Skeletal Fractures in Postmenopausal Women With and Without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose Tissue-Derived Mesenchymal Stem Cells In Vitro. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2016;31(12):2173-92.
272. Weilner S, Skalicky S, Salzer B, Keider V, Wagner M, Hildner F, et al. Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation. Bone. 2015;79:43-51.

273. Kocijan R, Muschitz C, Geiger E, Skalicky S, Baierl A, Dormann R, et al. Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures. The Journal of clinical endocrinology and metabolism. 2016;101(11):4125-34.
274. Walter E, Dellago H, Grillari J, Dimai HP, Hackl M. Cost-utility analysis of fracture risk assessment using microRNAs compared with standard tools and no monitoring in the Austrian female population. Bone. 2018;108:44-54.
275. Willeit P, Zampetaki A, Dudek K, Kaudewitz D, King A, Kirkby NS, et al. Circulating microRNAs as novel biomarkers for platelet activation. Circulation research. 2013;112(4):595-600.
276. Krammer TL, Mayr M, Hackl M. microRNAs as promising biomarkers of platelet activity in antiplatelet therapy monitoring. Int J Mol Sci. 2020;21(10).
277. Sheinerman KS, Tsivinsky VG, Abdullah L, Crawford F, Umansky SR. Plasma microRNA biomarkers for detection of mild cognitive impairment: biomarker validation study. Aging. 2013;5(12):925-38.
278. Sheinerman KS, Toledo JB, Tsivinsky VG, Irwin D, Grossman M, Weintraub D, et al. Circulating brain-enriched microRNAs as novel biomarkers for detection and differentiation of neurodegenerative diseases. Alzheimer's research & therapy. 2017;9(1):89.
279. López-Longarela B, Morrison EE, Tranter JD, Chahman-Vos L, Léonard JF, Gautier JC, et al. Direct Detection of miR-122 in Hepatotoxicity Using Dynamic Chemical Labeling Overcomes Stability and isomiR Challenges. Analytical chemistry. 2020;92(4):3388-95.
280. Marín-Romero A, Robles-Remacho A, Tabraue-Chávez M, López-Longarela B, Sánchez-Martín RM, Guardia-Monteagudo JJ, et al. A PCR-free technology to detect and quantify microRNAs directly from human plasma. The Analyst. 2018;143(23):5676-82.
281. Witwer KW, Halushka MK. Toward the promise of microRNAs - Enhancing reproducibility and rigor in microRNA research. RNA Biol. 2016;13(11):1103-16.
282. Lu Q, Wu R, Zhao M, Garcia-Gomez A, Ballestar E. miRNAs as Therapeutic Targets in Inflammatory Disease. Trends in Pharmacological Sciences. 2019;40(11):853-65.
283. Paul S, Bravo Vázquez LA, Pérez Uribe S, Roxana Reyes-Pérez P, Sharma A. Current Status of microRNA-Based Therapeutic Approaches in Neurodegenerative Disorders. Cells. 2020;9(7).
284. Bajan S, Hutvagner G. RNA-Based Therapeutics: From Antisense Oligonucleotides to miRNAs. Cells. 2020;9(1).
285. Krützfeldt J. Strategies to use microRNAs as therapeutic targets. Best Practice & Research Clinical Endocrinology & Metabolism. 2016;30(5):551-61.
286. Li Z, Rana TM. Therapeutic targeting of microRNAs: current status and future challenges. Nature Reviews Drug Discovery. 2014;13(8):622-38.

287. Krützfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438(7068):685-9.
288. Janssen HLA, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al. Treatment of HCV Infection by Targeting MicroRNA. New England Journal of Medicine. 2013;368(18):1685-94.
289. Gebert LFR, Rebhan MAE, Crivelli SEM, Denzler R, Stoffel M, Hall J. Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. Nucleic acids research. 2014;42(1):609-21.
290. Ottosen S, Parsley TB, Yang L, Zeh K, van Doorn L-J, van der Veer E, et al. In Vitro Antiviral Activity and Preclinical and Clinical Resistance Profile of Miravirsen, a Novel Anti-Hepatitis C Virus Therapeutic Targeting the Human Factor miR-122. Antimicrobial Agents and Chemotherapy. 2015;59(1):599-608.
291. Reesink HW, Janssen HLA, Zeuzem S, Lawitz E, Rodriguez-Torres M, Patel K, et al. Randomized, double-blind, placebo-controlled safety, anti-viral proof-of-concept study of Miravirsen, an oligonucleotide targeting miR-122, in treatment-naive patients with genotype 1 (gt1) chronic HCV infection. Journal of Hepatology. 2012;56:S26.
292. van der Ree MH, van der Meer AJ, van Nuenen AC, de Bruijne J, Ottosen S, Janssen HL, et al. Miravirsen dosing in chronic hepatitis C patients results in decreased microRNA-122 levels without affecting other microRNAs in plasma. Alimentary Pharmacology & Therapeutics. 2016;43(1):102-13.
293. Gallant-Behm CL, Piper J, Lynch JM, Seto AG, Hong SJ, Mustoe TA, et al. A MicroRNA-29 Mimic (Remlarsen) Represses Extracellular Matrix Expression and Fibroplasia in the Skin. The Journal of investigative dermatology. 2019;139(5):1073-81.
294. Cushing L, Kuang P, Lü J. The role of miR-29 in pulmonary fibrosis. Biochemistry and cell biology = Biochimie et biologie cellulaire. 2015;93(2):109-18.
295. Harmanci D, Erkan EP, Kocak A, Akdogan GG. Role of the microRNA-29 family in fibrotic skin diseases. Biomedical reports. 2017;6(6):599-604.
296. Seto AG, Beatty X, Lynch JM, Hermreck M, Tetzlaff M, Duvic M, et al. Cobomarsen, an oligonucleotide inhibitor of miR-155, co-ordinately regulates multiple survival pathways to reduce cellular proliferation and survival in cutaneous T-cell lymphoma. British journal of haematology. 2018;183(3):428-44.
297. Reid G, Pel ME, Kirschner MB, Cheng YY, Mugridge N, Weiss J, et al. Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma. Annals of oncology : official journal of the European Society for Medical Oncology. 2013;24(12):3128-35.
298. van Zandwijk N, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S, et al. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural

mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. The Lancet Oncology. 2017;18(10):1386-96.
299. Vermeire S, Hébuterne X, Napora P, Wisniewska-Jarosinska M, Kiss G, Bourreille A, et al. OP21 ABX464 is safe and efficacious in a proof-of-concept study in ulcerative colitis patients. Journal of Crohn's and Colitis. 2019;13(Supplement_1):S014-S5.
300. Vermeire S, Hébuterne X, Tilg H, De Hertogh G, Gineste P, Steens J-M. Induction and long-term follow-up with ABX464 for moderate-to-severe ulcerative colitis: Results of phase 2a trial. Gastroenterology. 2021.
301. Nejad C, Stunden HJ, Gantier MP. A guide to miRNAs in inflammation and innate immune responses. The FEBS journal. 2018;285(20):3695-716.
302. O'Neill LA, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat Rev Immunol. 2011;11(3):163-75.
303. Tahamtan A, Teymoori-Rad M, Nakstad B, Salimi V. Anti-Inflammatory MicroRNAs and Their Potential for Inflammatory Diseases Treatment. Frontiers in immunology. 2018;9:1377-.
304. Momen-Heravi F, Bala S. miRNA regulation of innate immunity. Journal of leukocyte biology. 2018;103(6):1205-17.
305. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature. 2008;454(7200):56-61.
306. Wiesen JL, Tomasi TB. Dicer is regulated by cellular stresses and interferons. Molecular immunology. 2009;46(6):1222-8.
307. Contreras J, Rao DS. MicroRNAs in inflammation and immune responses. Leukemia. 2012;26(3):404-13.
308. Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. Cell. 2009;136(1):26-36.
309. Sugimoto MA, Sousa LP, Pinho V, Perretti M, Teixeira MM. Resolution of Inflammation: What Controls Its Onset? Frontiers in Immunology. 2016;7(160).
310. Silva AM, Almeida MI, Teixeira JH, Ivan C, Oliveira J, Vasconcelos D, et al. Profiling the circulating miRnome reveals a temporal regulation of the bone injury response. Theranostics. 2018;8(14):3902-17.
311. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, et al. Chronic inflammation in the etiology of disease across the life span. Nat Med. 2019;25(12):1822-32.
312. Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, et al. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. Proc Natl Acad Sci U S A. 2009;106(13):5282-7.
313. Wang Y, Han Z, Fan Y, Zhang J, Chen K, Gao L, et al. MicroRNA-9 Inhibits NLRP3 Inflammasome Activation in Human Atherosclerosis Inflammation Cell Models through the

JAK1/STAT Signaling Pathway. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2017;41(4):1555-71.
314. Zhang Y, Jia J, Yang S, Liu X, Ye S, Tian H. MicroRNA-21 controls the development of osteoarthritis by targeting GDF-5 in chondrocytes. Experimental & molecular medicine. 2014;46:e79.
315. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. Journal of immunology (Baltimore, Md : 1950). 2009;182(8):4994-5002.
316. Cheng Y, Zhang C. MicroRNA-21 in cardiovascular disease. Journal of cardiovascular translational research. 2010;3(3):251-5.
317. Sheedy FJ. Turning 21: Induction of miR-21 as a Key Switch in the Inflammatory Response. Frontiers in immunology. 2015;6:19-.
318. Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, et al. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. Nat Immunol. 2010;11(2):141-7.
319. Xiao Y-T, Wang J, Lu W, Cao Y, Cai W. Downregulated expression of microRNA-124 in pediatric intestinal failure patients modulates macrophages activation by inhibiting STAT3 and AChE. Cell Death & Disease. 2016;7(12):e2521-e.
320. Ma C, Li Y, Li M, Deng G, Wu X, Zeng J, et al. microRNA-124 negatively regulates TLR signaling in alveolar macrophages in response to mycobacterial infection. Molecular immunology. 2014;62(1):150-8.
321. Sun Y, Li Q, Gui H, Xu D-P, Yang Y-L, Su D-F, et al. MicroRNA-124 mediates the cholinergic anti-inflammatory action through inhibiting the production of pro-inflammatory cytokines. Cell Research. 2013;23(11):1270-83.
322. Nakamachi Y, Kawano S, Takenokuchi M, Nishimura K, Sakai Y, Chin T, et al. MicroRNA-124a is a key regulator of proliferation and monocyte chemoattractant protein 1 secretion in fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis and rheumatism. 2009;60(5):1294-304.
323. Wang D, Zhang H, Li M, Frid MG, Flockton AR, McKeon BA, et al. MicroRNA-124 controls the proliferative, migratory, and inflammatory phenotype of pulmonary vascular fibroblasts. Circulation research. 2014;114(1):67-78.
324. Koukos G, Polytarchou C, Kaplan JL, Morley-Fletcher A, Gras-Miralles B, Kokkotou E, et al. MicroRNA-124 regulates STAT3 expression and is down-regulated in colon tissues of pediatric patients with ulcerative colitis. Gastroenterology. 2013;145(4):842-52.e2.
325. Banerjee S, Cui H, Xie N, Tan Z, Yang S, Icyuz M, et al. miR-125a-5p regulates differential activation of macrophages and inflammation. The Journal of biological chemistry. 2013;288(49):35428-36.

326. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. Journal of immunology (Baltimore, Md : 1950). 2007;179(8):5082-9.
327. Murphy AJ, Guyre PM, Pioli PA. Estradiol suppresses NF-kappa B activation through coordinated regulation of let-7a and miR-125b in primary human macrophages. Journal of immunology (Baltimore, Md : 1950). 2010;184(9):5029-37.
328. Sun CM, Wu J, Zhang H, Shi G, Chen ZT. Circulating miR-125a but not miR-125b is decreased in active disease status and negatively correlates with disease severity as well as inflammatory cytokines in patients with Crohn's disease. World journal of gastroenterology. 2017;23(44):7888-98.
329. Zhao X, Tang Y, Qu B, Cui H, Wang S, Wang L, et al. MicroRNA-125a contributes to elevated inflammatory chemokine RANTES levels via targeting KLF13 in systemic lupus erythematosus. Arthritis and rheumatism. 2010;62(11):3425-35.
330. Taganov KD, Boldin MP, Chang K-J, Baltimore D. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proceedings of the National Academy of Sciences. 2006;103(33):12481-6.
331. Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, et al. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. Mol Endocrinol. 2009;23(11):1876-84.
332. Shaked I, Meerson A, Wolf Y, Avni R, Greenberg D, Gilboa-Geffen A, et al. MicroRNA-132 Potentiates Cholinergic Anti-Inflammatory Signaling by Targeting Acetylcholinesterase. Immunity. 2009;31(6):965-73.
333. Maharshak N, Shenhar-Tsarfaty S, Aroyo N, Orpaz N, Guberman I, Canaani J, et al. MicroRNA-132 Modulates Cholinergic Signaling and Inflammation in Human Inflammatory Bowel Disease. Inflammatory bowel diseases. 2013;19(7):1346-53.
334. Starczynowski DT, Kuchenbauer F, Argiropoulos B, Sung S, Morin R, Muranyi A, et al. Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype. Nat Med. 2010;16(1):49-58.
335. O'Leary L, Sevinç K, Papazoglou IM, Tildy B, Detillieux K, Halayko AJ, et al. Airway smooth muscle inflammation is regulated by microRNA-145 in COPD. FEBS letters. 2016;590(9):1324-34.
336. Yuan M, Zhang L, You F, Zhou J-y, Ma Y, Yang F, et al. MiR-145-5p regulates hypoxia-induced inflammatory response and apoptosis in cardiomyocytes by targeting CD40. Molecular and Cellular Biochemistry. 2017;431:123-31.

337. Hu G, Zhao X, Wang C, Geng Y, Zhao J, Xu J, et al. MicroRNA-145 attenuates TNF-α-driven cartilage matrix degradation in osteoarthritis via direct suppression of MKK4. Cell Death & Disease. 2017;8(10):e3140-e.
338. Nahid MA, Satoh M, Chan EK. Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. Journal of immunology (Baltimore, Md : 1950). 2011;186(3):1723-34.
339. Park H, Huang X, Lu C, Cairo MS, Zhou X. MicroRNA-146a and microRNA-146b regulate human dendritic cell apoptosis and cytokine production by targeting TRAF6 and IRAK1 proteins. The Journal of biological chemistry. 2015;290(5):2831-41.
340. Curtale G, Citarella F, Carissimi C, Goldoni M, Carucci N, Fulci V, et al. An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes. Blood. 2010;115(2):265-73.
341. Jurkin J, Schichl YM, Koeffel R, Bauer T, Richter S, Konradi S, et al. miR-146a is differentially expressed by myeloid dendritic cell subsets and desensitizes cells to TLR2-dependent activation. Journal of immunology (Baltimore, Md : 1950). 2010;184(9):4955-65.
342. Hijmans JG, Diehl KJ, Bammert TD, Kavlich PJ, Lincenberg GM, Greiner JJ, et al. Influence of Overweight and Obesity on Circulating Inflammation-Related microRNA. MicroRNA (Shariqah, United Arab Emirates). 2018;7(2):148-54.
343. Hijmans JG, Diehl KJ, Bammert TD, Kavlich PJ, Lincenberg GM, Greiner JJ, et al. Association between hypertension and circulating vascular-related microRNAs. Journal of human hypertension. 2018;32(6):440-7.
344. Cardoso AL, Guedes JR, Pereira de Almeida L, Pedroso de Lima MC. miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. Immunology. 2012;135(1):73-88.
345. Li X, Kong D, Chen H, Liu S, Hu H, Wu T, et al. miR-155 acts as an anti-inflammatory factor in atherosclerosis-associated foam cell formation by repressing calcium-regulated heat stable protein 1. Scientific Reports. 2016;6(1):21789.
346. Yuan K, Zhang X, Lv L, Zhang J, Liang W, Wang P. Fine-tuning the expression of microRNA-155 controls acetaminophen-induced liver inflammation. International immunopharmacology. 2016;40:339-46.
347. Xie W, Li M, Xu N, Lv Q, Huang N, He J, et al. MiR-181a regulates inflammation responses in monocytes and macrophages. PloS one. 2013;8(3):e58639.
348. Zhu J, Wang F-L, Wang H-B, Dong N, Zhu X-M, Wu Y, et al. TNF-α mRNA is negatively regulated by microRNA-181a-5p in maturation of dendritic cells induced by high mobility group box-1 protein. Scientific Reports. 2017;7(1):12239.

349. Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, et al. MicroRNA-181b regulates NF-κB–mediated vascular inflammation. The Journal of Clinical Investigation. 2012;122(6):1973-90.
350. Hutchison ER, Kawamoto EM, Taub DD, Lal A, Abdelmohsen K, Zhang Y, et al. Evidence for miR-181 involvement in neuroinflammatory responses of astrocytes. Glia. 2013;61(7):1018-28.
351. Zhang L, Dong L-Y, Li Y-J, Hong Z, Wei W-S. The microRNA miR-181c controls microglia-mediated neuronal apoptosis by suppressing tumor necrosis factor. Journal of neuroinflammation. 2012;9(1):211.
352. Li T, Morgan MJ, Choksi S, Zhang Y, Kim YS, Liu ZG. MicroRNAs modulate the noncanonical transcription factor NF-kappaB pathway by regulating expression of the kinase IKKalpha during macrophage differentiation. Nat Immunol. 2010;11(9):799-805.
353. Chen Q, Wang H, Liu Y, Song Y, Lai L, Han Q, et al. Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3. PloS one. 2012;7(8):e42971-e.
354. Zhuang G, Meng C, Guo X, Cheruku PS, Shi L, Xu H, et al. A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation. Circulation. 2012;125(23):2892-903.
355. Haneklaus M, Gerlic M, Kurowska-Stolarska M, Rainey AA, Pich D, McInnes IB, et al. Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1β production. Journal of immunology (Baltimore, Md : 1950). 2012;189(8):3795-9.
356. Polytarchou C, Oikonomopoulos A, Mahurkar S, Touroutoglou A, Koukos G, Hommes DW, et al. Assessment of Circulating MicroRNAs for the Diagnosis and Disease Activity Evaluation in Patients with Ulcerative Colitis by Using the Nanostring Technology. Inflammatory bowel diseases. 2015;21(11):2533-9.
357. Neudecker V, Haneklaus M, Jensen O, Khailova L, Masterson JC, Tye H, et al. Myeloid-derived miR-223 regulates intestinal inflammation via repression of the NLRP3 inflammasome. The Journal of experimental medicine. 2017;214(6):1737-52.
358. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature. 2000;408(6808):86-9.
359. Teng GG, Wang WH, Dai Y, Wang SJ, Chu YX, Li J. Let-7b is involved in the inflammation and immune responses associated with Helicobacter pylori infection by targeting Toll-like receptor 4. PloS one. 2013;8(2):e56709.
360. Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, et al. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity. 2009;31(2):220-31.

361. Chen X-M, Splinter PL, O'Hara SP, LaRusso NF. A Cellular Micro-RNA, let-7i, Regulates Toll-like Receptor 4 Expression and Contributes to Cholangiocyte Immune Responses against Cryptosporidium parvum Infection. Journal of Biological Chemistry. 2007;282(39):28929-38.
362. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell. 2009;139(4):693-706.
363. Banerjee S, Xie N, Cui H, Tan Z, Yang S, Icyuz M, et al. MicroRNA let-7c regulates macrophage polarization. Journal of immunology (Baltimore, Md : 1950). 2013;190(12):6542-9.
364. Kumar M, Sahu SK, Kumar R, Subuddhi A, Maji RK, Jana K, et al. MicroRNA let-7 modulates the immune response to Mycobacterium tuberculosis infection via control of A20, an inhibitor of the NF-κB pathway. Cell host & microbe. 2015;17(3):345-56.
365. Pape K, Tamouza R, Leboyer M, Zipp F. Immunoneuropsychiatry — novel perspectives on brain disorders. Nature Reviews Neurology. 2019;15(6):317-28.
366. DiSabato DJ, Quan N, Godbout JP. Neuroinflammation: the devil is in the details. Journal of Neurochemistry. 2016;139(S2):136-53.
367. Lloyd AF, Miron VE. The pro-remyelination properties of microglia in the central nervous system. Nature Reviews Neurology. 2019;15(8):447-58.
368. Tremblay M-È, Stevens B, Sierra A, Wake H, Bessis A, Nimmerjahn A. The Role of Microglia in the Healthy Brain. The Journal of Neuroscience. 2011;31(45):16064-9.
369. Wake H, Fields RD. Physiological function of microglia. Neuron glia biology. 2011;7(1):1-3.
370. Ponomarev ED, Veremeyko T, Weiner HL. MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. Glia. 2013;61(1):91-103.
371. Ponomarev ED, Veremeyko T, Barteneva N, Krichevsky AM, Weiner HL. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. Nat Med. 2011;17.
372. Song J, Oh Y, Lee JE. miR-Let7A Modulates Autophagy Induction in LPS-Activated Microglia. Exp Neurobiol. 2015;24(2):117-25.
373. Shi G, Shi J, Liu K, Liu N, Wang Y, Fu Z, et al. Increased miR-195 aggravates neuropathic pain by inhibiting autophagy following peripheral nerve injury. Glia. 2013;61(4):504-12.
374. Yu A, Zhang T, Zhong W, Duan H, Wang S, Ye P, et al. miRNA-144 induces microglial autophagy and inflammation following intracerebral hemorrhage. Immunology Letters. 2017;182:18-23.

375. Li Y, Zhou D, Ren Y, Zhang Z, Guo X, Ma M, et al. Mir223 restrains autophagy and promotes CNS inflammation by targeting ATG16L1. Autophagy. 2019;15(3):478-92.
376. Heppner FL, Ransohoff RM, Becher B. Immune attack: the role of inflammation in Alzheimer disease. Nature Reviews Neuroscience. 2015;16(6):358-72.
377. Paresce DM, Ghosh RN, Maxfield FR. Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. Neuron. 1996;17(3):553-65.
378. Hansen DV, Hanson JE, Sheng M. Microglia in Alzheimer's disease. J Cell Biol. 2018;217(2):459-72.
379. Efthymiou AG, Goate AM. Late onset Alzheimer's disease genetics implicates microglial pathways in disease risk. Molecular neurodegeneration. 2017;12(1):43-.
380. Aloi MS, Prater KE, Sopher B, Davidson S, Jayadev S, Garden GA. The pro-inflammatory microRNA miR-155 influences fibrillar β-Amyloid1-42 catabolism by microglia. Glia. 2021;69(7):1736-48.
381. Jiang W, Zhang Y, Meng F, Lian B, Chen X, Yu X, et al. Identification of active transcription factor and miRNA regulatory pathways in Alzheimer's disease. Bioinformatics. 2013;29(20):2596-602.
382. Takousis P, Sadlon A, Schulz J, Wohlers I, Dobricic V, Middleton L, et al. Differential expression of microRNAs in Alzheimer's disease brain, blood, and cerebrospinal fluid. Alzheimer's & dementia : the journal of the Alzheimer's Association. 2019;15(11):1468-77.
383. Li YY, Cui JG, Dua P, Pogue AI, Bhattacharjee S, Lukiw WJ. Differential expression of miRNA-146a-regulated inflammatory genes in human primary neural, astroglial and microglial cells. Neurosci Lett. 2011;499(2):109-13.
384. Lukiw WJ, Alexandrov PN. Regulation of complement factor H (CFH) by multiple miRNAs in Alzheimer's disease (AD) brain. Mol Neurobiol. 2012;46(1):11-9.
385. Lukiw WJ, Zhao Y, Cui JG. An NF-kappaB-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. The Journal of biological chemistry. 2008;283(46):31315-22.
386. Jayadev S, Case A, Alajajian B, Eastman AJ, Möller T, Garden GA. Presenilin 2 influences miR146 level and activity in microglia. Journal of neurochemistry. 2013;127(5):592-9.
387. Saba R, Gushue S, Huzarewich RLCH, Manguiat K, Medina S, Robertson C, et al. MicroRNA 146a (miR-146a) Is Over-Expressed during Prion Disease and Modulates the Innate Immune Response and the Microglial Activation State. PloS one. 2012;7(2):e30832.
388. Bhattacharjee S, Zhao Y, Lukiw WJ. Deficits in the miRNA-34a-regulated endogenous TREM2 phagocytosis sensor-receptor in Alzheimer's disease (AD); an update. Frontiers in aging neuroscience. 2014;6:116-.

389. Zhao Y, Bhattacharjee S, Jones BM, Hill J, Dua P, Lukiw WJ. Regulation of Neurotropic Signaling by the Inducible, NF-κB-Sensitive miRNA-125b in Alzheimer's Disease (AD) and in Primary Human Neuronal-Glial (HNG) Cells. Molecular Neurobiology. 2014;50(1):97-106.
390. Tan E-K, Chao Y-X, West A, Chan L-L, Poewe W, Jankovic J. Parkinson disease and the immune system — associations, mechanisms and therapeutics. Nature Reviews Neurology. 2020;16(6):303-18.
391. Bai X, Tang Y, Yu M, Wu L, Liu F, Ni J, et al. Downregulation of blood serum microRNA 29 family in patients with Parkinson's disease. Sci Rep. 2017;7(1):5411.
392. Wang R, Yang Y, Wang H, He Y, Li C. MiR-29c protects against inflammation and apoptosis in Parkinson's disease model in vivo and in vitro by targeting SP1. Clinical and experimental pharmacology & physiology. 2020;47(3):372-82.
393. Wang R, Li Q, He Y, Yang Y, Ma Q, Li C. miR-29c-3p inhibits microglial NLRP3 inflammasome activation by targeting NFAT5 in Parkinson's disease. Genes to Cells. 2020;25(6):364-74.
394. Briggs CE, Wang Y, Kong B, Woo TU, Iyer LK, Sonntag KC. Midbrain dopamine neurons in Parkinson's disease exhibit a dysregulated miRNA and target-gene network. Brain Res. 2015;1618:111-21.
395. Tatura R, Kraus T, Giese A, Arzberger T, Buchholz M, Höglinger G, et al. Parkinson's disease: SNCA-, PARK2-, and LRRK2- targeting microRNAs elevated in cingulate gyrus. Parkinsonism & related disorders. 2016;33:115-21.
396. Martins M, Rosa A, Guedes LC, Fonseca BV, Gotovac K, Violante S, et al. Convergence of miRNA expression profiling, α-synuclein interacton and GWAS in Parkinson's disease. PloS one. 2011;6(10):e25443.
397. Chen L, Yang J, Lü J, Cao S, Zhao Q, Yu Z. Identification of aberrant circulating miRNAs in Parkinson's disease plasma samples. Brain and behavior. 2018;8(4):e00941.
398. Li D, Yang H, Ma J, Luo S, Chen S, Gu Q. MicroRNA-30e regulates neuroinflammation in MPTP model of Parkinson's disease by targeting Nlrp3. Human cell. 2018;31(2):106-15.
399. Zhao J, Zhou Y, Guo M, Yue D, Chen C, Liang G, et al. MicroRNA-7: expression and function in brain physiological and pathological processes. Cell & Bioscience. 2020;10(1):77.
400. Angelopoulou E, Paudel YN, Piperi C. miR-124 and Parkinson's disease: A biomarker with therapeutic potential. Pharmacological research. 2019;150:104515.
401. Junn E, Lee K-W, Jeong BS, Chan TW, Im J-Y, Mouradian MM. Repression of α-synuclein expression and toxicity by microRNA-7. Proceedings of the National Academy of Sciences. 2009;106(31):13052-7.
402. Yao L, Ye Y, Mao H, Lu F, He X, Lu G, et al. MicroRNA-124 regulates the expression of MEKK3 in the inflammatory pathogenesis of Parkinson's disease. Journal of neuroinflammation. 2018;15(1):13-.

403. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. Nature Reviews Immunology. 2015;15(9):545-58.
404. Lopez-Ramirez MA, Wu D, Pryce G, Simpson JE, Reijerkerk A, King-Robson J, et al. MicroRNA-155 negatively affects blood-brain barrier function during neuroinflammation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2014;28(6):2551-65.
405. Junker A, Krumbholz M, Eisele S, Mohan H, Augstein F, Bittner R, et al. MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. Brain. 2009;132(Pt 12):3342-52.
406. Talebi F, Ghorbani S, Chan WF, Boghozian R, Masoumi F, Ghasemi S, et al. MicroRNA-142 regulates inflammation and T cell differentiation in an animal model of multiple sclerosis. Journal of neuroinflammation. 2017;14(1):55.
407. Müller N, Weidinger E, Leitner B, Schwarz MJ. The role of inflammation in schizophrenia. Frontiers in neuroscience. 2015;9:372-.
408. Volk DW, Chitrapu A, Edelson JR, Roman KM, Moroco AE, Lewis DA. Molecular mechanisms and timing of cortical immune activation in schizophrenia. The American journal of psychiatry. 2015;172(11):1112-21.
409. Radewicz K, Garey LJ, Gentleman SM, Reynolds R. Increase in HLA-DR immunoreactive microglia in frontal and temporal cortex of chronic schizophrenics. Journal of neuropathology and experimental neurology. 2000;59(2):137-50.
410. Wierzba-Bobrowicz T, Lewandowska E, Lechowicz W, Stepień T, Pasennik E. Quantitative analysis of activated microglia, ramified and damage of processes in the frontal and temporal lobes of chronic schizophrenics. Folia neuropathologica. 2005;43(2):81-9.
411. Steiner J, Bielau H, Brisch R, Danos P, Ullrich O, Mawrin C, et al. Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide. Journal of psychiatric research. 2008;42(2):151-7.
412. Trépanier MO, Hopperton KE, Mizrahi R, Mechawar N, Bazinet RP. Postmortem evidence of cerebral inflammation in schizophrenia: a systematic review. Molecular psychiatry. 2016;21(8):1009-26.
413. Kim AH, Reimers M, Maher B, Williamson V, McMichael O, McClay JL, et al. MicroRNA expression profiling in the prefrontal cortex of individuals affected with schizophrenia and bipolar disorders. Schizophrenia Research. 2010;124(1):183-91.
414. Miller BH, Zeier Z, Xi L, Lanz TA, Deng S, Strathmann J, et al. MicroRNA-132 dysregulation in schizophrenia has implications for both neurodevelopment and adult brain function. Proceedings of the National Academy of Sciences. 2012;109(8):3125-30.

415. Perkins DO, Jeffries CD, Jarskog LF, Thomson JM, Woods K, Newman MA, et al. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. Genome biology. 2007;8(2):R27.
416. Beveridge NJ, Tooney PA, Carroll AP, Gardiner E, Bowden N, Scott RJ, et al. Dysregulation of miRNA 181b in the temporal cortex in schizophrenia. Human Molecular Genetics. 2008;17(8):1156-68.
417. Shi W, Du J, Qi Y, Liang G, Wang T, Li S, et al. Aberrant expression of serum miRNAs in schizophrenia. Journal of psychiatric research. 2012;46(2):198-204.
418. Liu S, Zhang F, Wang X, Shugart YY, Zhao Y, Li X, et al. Diagnostic value of blood-derived microRNAs for schizophrenia: results of a meta-analysis and validation. Scientific Reports. 2017;7(1):15328.
419. Goldsmith DR, Rapaport MH, Miller BJ. A meta-analysis of blood cytokine network alterations in psychiatric patients: comparisons between schizophrenia, bipolar disorder and depression. Molecular psychiatry. 2016;21(12):1696-709.
420. Rao JS, Harry GJ, Rapoport SI, Kim HW. Increased excitotoxicity and neuroinflammatory markers in postmortem frontal cortex from bipolar disorder patients. Molecular psychiatry. 2010;15(4):384-92.
421. Haarman BC, Riemersma-Van der Lek RF, de Groot JC, Ruhé HG, Klein HC, Zandstra TE, et al. Neuroinflammation in bipolar disorder - A [(11)C]-(R)-PK11195 positron emission tomography study. Brain, behavior, and immunity. 2014;40:219-25.
422. Moreau MP, Bruse SE, David-Rus R, Buyske S, Brzustowicz LM. Altered microRNA expression profiles in postmortem brain samples from individuals with schizophrenia and bipolar disorder. Biological psychiatry. 2011;69(2):188-93.
423. Azevedo JA, Carter BS, Meng F, Turner DL, Dai M, Schatzberg AF, et al. The microRNA network is altered in anterior cingulate cortex of patients with unipolar and bipolar depression. Journal of psychiatric research. 2016;82:58-67.
424. Bavamian S, Mellios N, Lalonde J, Fass DM, Wang J, Sheridan SD, et al. Dysregulation of miR-34a links neuronal development to genetic risk factors for bipolar disorder. Molecular psychiatry. 2015;20(5):573-84.
425. Smalheiser NR, Lugli G, Zhang H, Rizavi H, Cook EH, Dwivedi Y. Expression of microRNAs and other small RNAs in prefrontal cortex in schizophrenia, bipolar disorder and depressed subjects. PloS one. 2014;9(1):e86469.
426. Geaghan M, Cairns MJ. MicroRNA and Posttranscriptional Dysregulation in Psychiatry. Biological psychiatry. 2015;78(4):231-9.
427. Smalheiser NR, Lugli G, Rizavi HS, Torvik VI, Turecki G, Dwivedi Y. MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects. PloS one. 2012;7(3):e33201-e.

428. Lopez JP, Fiori LM, Gross JA, Labonte B, Yerko V, Mechawar N, et al. Regulatory role of miRNAs in polyamine gene expression in the prefrontal cortex of depressed suicide completers. The international journal of neuropsychopharmacology. 2014;17(1):23-32.
429. Sun N, Lei L, Wang Y, Yang C, Liu Z, Li X, et al. Preliminary comparison of plasma notch-associated microRNA-34b and -34c levels in drug naive, first episode depressed patients and healthy controls. Journal of affective disorders. 2016;194:109-14.
430. Pan B, Liu Y. Effects of duloxetine on microRNA expression profile in frontal lobe and hippocampus in a mouse model of depression. International journal of clinical and experimental pathology. 2015;8(11):15454-61.
431. Roy B, Dunbar M, Shelton RC, Dwivedi Y. Identification of MicroRNA-124-3p as a Putative Epigenetic Signature of Major Depressive Disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2017;42(4):864-75.
432. He S, Liu X, Jiang K, Peng D, Hong W, Fang Y, et al. Alterations of microRNA-124 expression in peripheral blood mononuclear cells in pre- and post-treatment patients with major depressive disorder. Journal of psychiatric research. 2016;78:65-71.
433. Li YJ, Xu M, Gao ZH, Wang YQ, Yue Z, Zhang YX, et al. Alterations of serum levels of BDNF-related miRNAs in patients with depression. PloS one. 2013;8(5):e63648.
434. Zhang H-p, Liu X-l, Chen J-j, Cheng K, Bai S-J, Zheng P, et al. Circulating microRNA 134 sheds light on the diagnosis of major depressive disorder. Translational psychiatry. 2020;10(1):95.
435. Lopez JP, Lim R, Cruceanu C, Crapper L, Fasano C, Labonte B, et al. miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment. Nat Med. 2014;20(7):764-8.
436. Fiori LM, Lopez JP, Richard-Devantoy S, Berlim M, Chachamovich E, Jollant F, et al. Investigation of miR-1202, miR-135a, and miR-16 in Major Depressive Disorder and Antidepressant Response. International Journal of Neuropsychopharmacology. 2017;20(8):619-23.
437. Bai M, Zhu X, Zhang Y, Zhang S, Zhang L, Xue L, et al. Abnormal hippocampal BDNF and miR-16 expression is associated with depression-like behaviors induced by stress during early life. PloS one. 2012;7(10):e46921.
438. Gheysarzadeh A, Sadeghifard N, Afraidooni L, Pooyan F, Mofid MR, Valadbeigi H, et al. Serum-based microRNA biomarkers for major depression: MiR-16, miR-135a, and miR-1202. J Res Med Sci. 2018;23:69-.
439. Rush AJ, Trivedi MH, Wisniewski SR, Nierenberg AA, Stewart JW, Warden D, et al. Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. The American journal of psychiatry. 2006;163(11):1905-17.

4. Aims of the thesis

The inflammatory theory of depression is widely accepted and researched, but it remains unknown if inflammation is the driving force or a consequence of depression. Moreover, molecular mechanisms underlying this link between inflammation and depression remain largely unexplored. miRNAs are key regulatory molecules which may allow us to look closer at the possible integration of the inflammatory process and depressive symptoms. Thus, the main goal of this PhD project was to understand how miRNAs can concomitantly impact both processes. Specifically, we aimed to:

1. *Explore the role of inflammation-related miRNAs in microglia activation and in microglia-to-neurons communication, through an in vitro approach.*

The aim of this task was to investigate the role of miRNAs in TNF-α-driven microglia activation, in order to identify potential therapeutic targets for microglia chronic activation and neuroinflammation. Specifically, we aimed to unravel how TNF-α impacted miRNAs expression, to identify which of these miRNAs are essential for microglia activation, ultimately determining their impact in the crosstalk with neurons. These results are presented in Chapter II.1.

2. *Understand how stress impacts inflammation-associated miRNAs in the brain, in vivo, using a rat model of stress inductor of depressive-like behaviours*

In this task, an *in vivo* rat model was used to explore how peripubertal stress combined with differential corticosterone-stress responsiveness influences depressive-like behaviours and the expression of inflammatory markers across different brain regions in adulthood. Moreover, we aimed to evaluate how these alterations correlate with microglia activation and the expression of inflammation-related miRNAs identified in task 1. These results are presented in Chapter II.2.

3. *Identify new biomarkers of depression in the miRNA profiles of circulating peripheral blood mononuclear cells of depression patients.*

In this task, we aimed to evaluate whether the levels of inflammatory cytokines in plasma and PBMCs and the expression levels of inflammation-related miRNAs in PBMCs, could be altered in depression patients compared with healthy controls. The potential of differently expressed miRNAs to be used as diagnostic markers of depression was tested. These results are presented in Chapter II.3.

II. Original research work

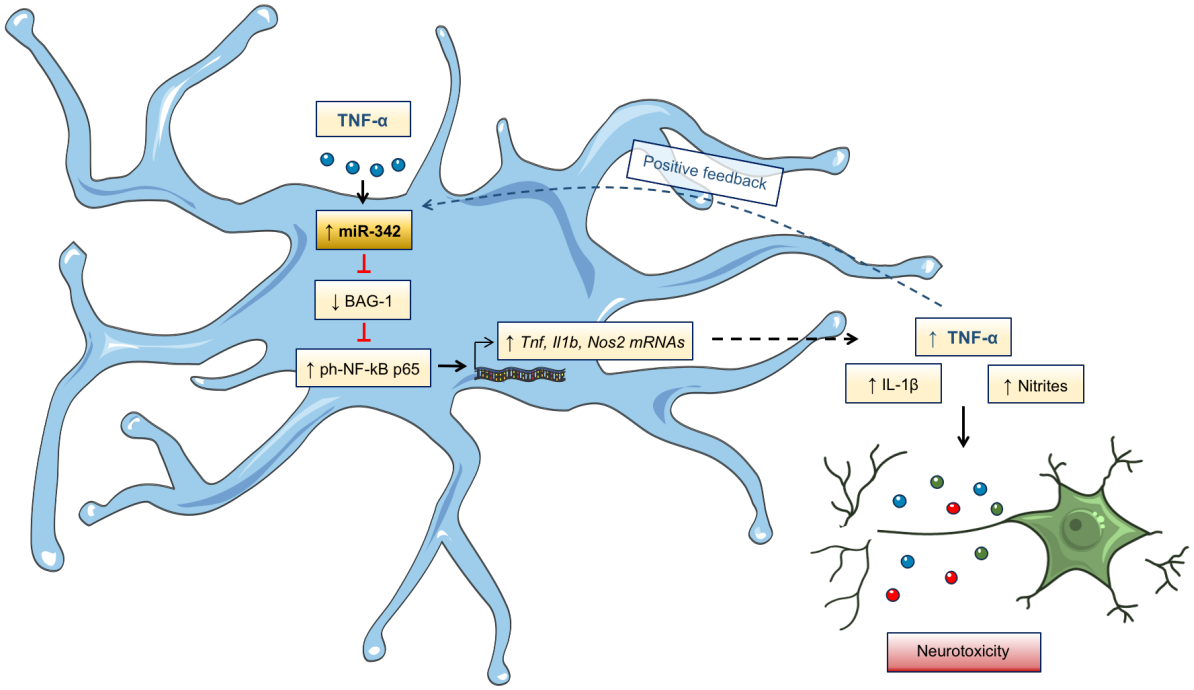
1. TNF-alpha-induced microglia activation requires miR-342: impact on NF-κB signalling and neurotoxicity

The content of this section is published in the following original research article:

Article II

João P. Brás, Joana Bravo, Jaime Freitas, Mário A. Barbosa, Susana G. Santos, Teresa Summavielle, Maria I. Almeida. **TNF-alpha-induced microglia activation requires miR-342: impact on NF-κB signalling and neurotoxicity.** Cell Death Dis. 2020 Jun 2;11(6):415. doi: 10.1038/s41419-020-2626-6.

Graphical abstract



Graphical abstract. TNF-α-induced miR-342 promotes microglia activation through NF-κB and induces neurotoxicity. We found miR-342 to be upregulated in microglia activated with TNF-α. miR-342 promotes NF-κB activation by inhibiting BAG-1, leading to the overexpression of pro-inflammatory mediators, including TNF-α, in a positive feedback loop, possibly perpetuating microglia activation. Importantly, inhibition of miR-342 attenuated TNF-α-driven microglia activation. Moreover, microglia activation by miR-342 led to increased neurotoxicity with high levels of nitrites being detected in co-cultures supernatants.

Abstract

Growing evidences suggest that sustained neuroinflammation, caused by microglia overactivation, is implicated in the development and aggravation of several neurological and psychiatric disorders. In some pathological conditions, microglia produce increased levels of cytotoxic and inflammatory mediators, such as TNF-α, which can reactivate microglia in a positive feedback mechanism. However, specific molecular mediators that can be effectively targeted to control TNF-α-mediated microglia overactivation, are yet to be uncovered. In this context, we aim to identify novel TNF-α mediated micro(mi)RNAs and to dissect their roles in microglia activation, as well as to explore their impact on the cellular communication with neurons. A miRNA microarray, followed by RT-qPCR validation, was performed on TNF-α-stimulated primary rat microglia. Gain- and loss-of-function *in vitro* assays and proteomic analysis were used to dissect the role of miR-342 in microglia activation. Co-cultures of microglia with hippocampal neurons, using a microfluidic system, were performed to understand the impact on neurotoxicity. Stimulation of primary rat microglia with TNF-α led to an upregulation of *Nos2*, *Tnf* and *Il1b* mRNAs. Additionally, ph-NF-κB p65 levels were also increased. miRNA microarray analysis followed by RT-qPCR validation revealed that TNF-α stimulation induced the upregulation of miR-342. Interestingly, miR-342 overexpression in N9 microglia was sufficient to activate the NF-κB pathway by inhibiting BAG-1, leading to increased secretion of TNF-α and IL-1β. Conversely, miR-342 inhibition led to a strong decrease in the levels of these cytokines after TNF-α activation. In fact, both TNF-α-stimulated and miR-342-overexpressing microglia drastically affected neuron viability. Remarkably, increased levels of nitrites were detected in the supernatants of these co-cultures. Globally, our findings show that miR-342 is a crucial mediator of TNF-α-mediated microglia activation and a potential target to tackle microglia-driven neuroinflammation.

Keywords: neuroinflammation, microglia, TNF-α, microRNA

1.1 Introduction

In recent years, the immune system has been associated with pathological events occurring in several neurodegenerative and psychiatric disorders (1). Accordingly, growing evidence shows that some of the patients suffering from these disorders have chronic microglia activation and overproduction of pro-inflammatory cytokines, such as tumour necrosis factor (TNF-α), which sustains several neuroinflammatory processes (2).

Microglia are the largest population of immune cells in the central nervous system (CNS), corresponding to 5 - 15% of all adult brain (3). Under physiological conditions, these cells are located within the brain parenchyma, where they are in direct contact with neural progenitors, neurons, and other glial cells (namely astrocytes and oligodendrocytes) (4). When an injury or infection takes place, microglia are recruited to the site where they engulf invading pathogens and extracellular debris by phagocytosis, supporting the normal function and integrity of the brain (5). In response to an inflammatory stimulus, microglia secrete a number of molecules such as proteinases, nitric oxide, reactive oxygen intermediates and pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and TNF-α (6, 7).

TNF-α is a major Th1-class pro-inflammatory cytokine, produced by a variety of immune cells. In the CNS, activated microglia are one of the main sources of TNF-α (8, 9). TNF-α can bind to TNFR1(p55) and/or TNFR2(p75), activating downstream signalling pathways that mediate a wide variety of biological responses, including apoptosis, cell differentiation, proliferation, survival and inflammation (10). At basal levels, TNF-α has an important role in brain development, particularly by influencing hippocampal development and function (11). However, in certain pathological conditions, increased levels of this cytokine overactivate microglia, which then causes neuronal damage, such as demyelination and/or neuronal degeneration. Overactivated microglia release cytotoxic molecules, including TNF-α, which is produced by a positive feedback mechanism of autocrine activation (12, 13). Although the basic mechanisms by which TNF-α activates microglia have been previously reported, the specific molecular mediators that can be effectively targeted to control TNF-α mediated microglia overactivation and neuroinflammation, are still to be uncovered.

In the last decade, microRNAs (miRNAs) have been extensively studied due to their regulatory role in many pathological events (14, 15). miRNAs are short, single-stranded, non-protein coding RNAs, that regulate gene expression at the post-transcriptional level by inducing mRNA translation inhibition or degradation (16). Importantly, miRNAs are highly conserved in mammalians (17) and are crucial players in key cellular processes, such as inflammation, cell death and differentiation. Remarkably, dysfunction in one single miRNA can concomitantly impair several biological functions by acting on distinct mRNA targets, as previously described by us (16-20). Additionally, miRNAs can be encapsulated into

extracellular vesicles, and mediate cell-to-cell communication (19, 21). All these features make miRNAs attractive candidates for use as biomarkers and/or as therapeutic targets.

Herein, we investigated the role of miRNAs in TNF-α-driven microglia activation, in order to identify potential therapeutic targets for microglia chronic activation and neuroinflammation. Specifically, we aimed to unravel how TNF-α impacted miRNAs expression, to identify which of these miRNAs are essential for microglia activation, ultimately determining their impact on the crosstalk between microglia and neurons.

1.2 Materials and methods

1.2.1 Animal ethical disclosure

All procedures to obtain primary cell cultures were conducted in accordance with European regulations (European Union Directive 2010/63/EU) and were approved by the i3S Animal Ethics Committee and the Portuguese regulatory entity — Direcção Geral de Alimentação e Veterinária (DGAV, ref 11769/2014-05-15 to TS). Animal facilities and the people directly involved in animal experiments (JPB and JB) were also certified. All efforts were made to ensure minimal animal suffering, and to follow the principles of the 3Rs.

1.2.2 Cell culture

Mixed glial cells. Briefly, Newburn Wistar Han Rats (P1-P2) were decapitated, and brains separated from the skull. Cerebellum and meninges were carefully removed for efficient brain dissection. Dissected tissue was treated with DNase and trypsin (Sigma, USA) before being dissociated and plated. Rat brain-derived mixed glial cells were cultured in T75 poly D-lysine (Sigma) coated flasks for 21 days. Culture was maintained in DMEM (Dulbecco's modified Eagle's medium; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, France).

Primary microglia. Primary microglia were isolated from rat mixed glial cultures using the shaking method, as previously described (22). Microglia were obtained after 14 and 21 days of culture by shaking the flasks at 37°C for 2h at 150 rpm. Isolated microglia were re-seeded ($3x10^4/cm^2$) in DMEM/F12 (Corning, USA) supplemented with 10% FBS, allowed to adhere for 48h and then stimulated for 6h with 100 ng/mL lipopolysaccharide (LPS, Sigma) or 20 ng/mL TNF-α (Peprotech, UK).

Primary hippocampal neurons. E17 C57BL/6 mice hippocampal neurons were cultured as previously described (23). Briefly, after dissection, hippocampi were treated with trypsin (1.5 mg/mL, 15 min, 37°C, Sigma) in Hank's balanced salt solution (HBSS; Gibco), washed with HBSS containing 10% FBS, to stop trypsin activity, and washed in HBSS to remove serum

and avoid glia growth. Finally, the tissue was transferred to serum-free Neurobasal medium (Gibco), supplemented with B27 (1:50, Gibco), glutamine (0.5 mM, Sigma), gentamycin (0.12 mg/mL Gibco) and glutamate (25 µM, Sigma), and dissociated mechanically. Neurons were then plated ($1x10^5$ cells/chamber) in poly-D-lysine-coated coverslips (20 µg/cm^2) previously attached to Axon Investigation Systems (AXIS™, AX150, Millipore) and maintained in the supplemented neurobasal medium. Cells were kept at 37°C in a humidified incubator with 5% CO_2/95% air, for 14 days, and half of the media was replaced at day 7.

N9 microglial cells. Murine N9 cells, kindly donated by Prof. João Relvas (i3S, Porto), were cultured in RPMI 1640 (Corning, USA) supplemented with 10% FBS in T75 flasks. All cell lines were tested and negative for mycoplasma contamination. Prior to transfections, cells were trypsinized, re-seeded ($1.5x10^4$/cm^2) and allowed to adhere for 24h. 70% confluent N9 cells were transfected with mirVana™ miRNA mimic/mirVana™ miRNA inhibitor for mmu-miR-342-3p, or respective controls (mirVana™ miRNA mimic/inhibitor negative controls; Invitrogen, USA), using Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. After transfection, when applicable, N9 cells were stimulated with 20 ng/mL of TNF-α for 6h. Conditioned media was collected for cytokine quantification and cells harvested for protein extraction or co-culture with neurons. Transfections with siRNA were also performed with Lipofectamine 2000. siRNA specific for murine BAG-1 (5′-CCGUUGUCAGCACUUGGAAUGCAAA-3') and siRNA negative control (12935-300) were purchased from Invitrogen. Overexpression of BAG-1 in N9 microglia was achieved by transiently transfecting the cells with a BAG-1 mammalian expression vector (pCMV6-BAG-1, Origene). Briefly, 24 hours before transfection, cells were seeded into 6-well cell culture plates at a density of $1.2x10^5$ cells per well in regular growth medium. Transfections were performed with Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). 48 hours after transfection, cells were harvested for the detection of ph-NF-κB p65 expression levels by western blot. The control vector pCMV6 was kindly donated by Mariana Santos (UnIGENe, i3S, Portugal).

Primary hippocampal neurons and N9 microglial cells co-culture. Co-cultures were performed in Axon Investigation System (Millipore). At day 13 of neuronal culture, transfected, TNF-α or non-stimulated N9 cells ($0.2x10^5$ cells) were added to each Axon Investigation System, in direct contact with axons for 24h. Culture media was then collected for nitrites quantification and cells were fixed prior to immunostaining.

1.2.3 Flow cytometry

Primary microglia culture purity was measured by flow cytometry using the following antibodies: mouse anti-rat CD11b/c-PE/Cy7 (BD Biosciences, USA) and mouse anti-rat CD45-FITC (ImmunoTools, Germany). Unlabelled microglia, mouse isotypes IgG2a-PE/Cy7

(BD Biosciences) and IgG2a-FITC (Immunotools) were used as negative controls. Fluorescence was measured in a FACS Canto II flow cytometer (BD Biosciences) with BD FACS Diva software. Results were analysed using FlowJo Software.

1.2.4 RNA extraction

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were evaluated in a NanoDrop 1000 (Thermo Scientific). Ratios of 260/280 and 260/230 nm ranged between 1.9 and 2.1. RNA integrity was evaluated by agarose gel electrophoresis or by Experion™ automated electrophoresis system (Bio-Rad, USA).

1.2.5 Reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR)

For gene expression analysis, RNA was treated with TURBO DNA-free Kit (Invitrogen) and cDNA was synthesized using Random Hexamers (Invitrogen), dNTPs (Bioline) and SuperScript® III Reverse Transcriptase (Invitrogen). qPCR was carried out in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) using cDNA, primers and iQ SYBR Green Supermix (Bio-Rad). Oligonucleotides used for qPCR experiments are shown in Supplementary Table 1.

miR-146b-5p, miR-342-3p, miR-124-3p and let-7i-5p expression was evaluated using TaqMan miRNA assays (Applied Biosystems). Briefly, cDNA was synthesized using 30 ng of RNA as a template, gene-specific stem-loop Reverse Transcription primer, and the TaqMan microRNA reverse transcription kit (Applied Biosystems). qPCR was carried out in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using cDNA, TaqMan probe and SsoAdvanced™ Universal Probes Supermix (Bio-Rad). Small nuclear RNA U6 was used as reference gene. All runs were performed in duplicate. Relative expression levels from 8 independent experiments were calculated using the quantification cycle (C_q) method, according to MIQE guidelines (24).

1.2.6 microRNA microarray assay

miRNA expression profile of TNF-α activated primary rat microglia was performed through ArrayStar, using a μParaflo™ mouse microRNA microarray, Array 19.0 (LC Sciences, USA). By adding a poly (A) tail to the 3' end with poly (A) polymerase, total RNA (2 μg/sample) was extended, and then an oligonucleotide tag was ligated to the poly (A) tail for fluorescent dye staining subsequently. Prepared RNA was hybridized to a μParaflo microfluidic chip. Detection probes were synthesized *in situ,* based on miRBase v22.0 database (www.mirbase.org) (25).

Following hybridization, the Cy3 dye was bound to the oligo tag for staining through the microfluidic chip. Fluorescence images were acquired with a laser scanner and digitized with Array-Pro image analysis software. Data relative to the fluorescent intensity of a given hybridization target (signal) is presented in arbitrary units (AU). Only miRNAs with average signals ≥ 20 and $-0.2 \leq \log_2$ fold change to CTR ≥ 0.2 were considered for further evaluation (Supplementary Table 3).

1.2.7 Western blot

N9 microglia cells were harvested and washed twice with cold PBS before lysis in the presence of protease and phosphatase inhibitors. Cell lysates were centrifuged (14000 rpm, 10 min, 4ºC) and total protein quantified using DC protein assay kit (Bio-Rad, USA). Protein samples were resolved by SDS-PAGE in reducing conditions and transferred to nitrocellulose membranes, which were blocked in a solution of 5% BSA in TBS-Tween 0.1%. Membranes were then probed using the following primary antibodies: anti-ph-NF-κB p65, anti-BAG-1, anti-α-Tubulin and anti-GAPDH. Appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) were used for signal detection. Antibodies manufacturers and respective dilutions are indicated in Supplementary Table 2. Protein expression levels were quantified using ImageLab. α-Tubulin and GAPDH were used as normalizers.

1.2.8 Analysis of nuclear NF-κB translocation by imaging flow cytometry

Murine N9 microglial cells were stimulated with 20 ng/mL of TNF-α for 10, 20 or 30 minutes or transfected with the miRNA mimic mmu-miR-342-3p and the miRNA mimic negative control (SCR), as described. Before harvesting, cells were washed twice with ice-cold PBS and fixed in 4% PFA. NF-κB staining with rabbit anti-mouse Phospho-NF-κB p65 (Ser536) (93H1) antibody (Cell Signaling Technologies) was performed in PBS with 0.1% Triton X-100 and 2% FBS for 20 min on ice, followed by a 20 min incubation with anti-rabbit Alexa Fluor 488-labeled secondary antibody (Thermo Fisher Scientific) in PBS with 2% FBS. Nuclei were stained with 20 mM DRAQ5 (Biostatus) for 10 min before acquisition. Cells were acquired using an ImageStreamX Imaging flow cytometer with the INSPIRE software and equipped with an Extended Depth of Field filter (Amnis, EMD Millipore), at the Bioimaging Center for Biomaterials and Regenerative Therapies (b.IMAGE – i3S). Data analysis was performed with IDEAS software (Amnis, EMD Millipore). Fluorescence compensation was performed with single stained samples. For each sample 30000 cells were acquired, and more than 10000 single, in focus, double positive for NF-κB and DRAQ5 cells were analysed for NF-κB translocalization. The IDEAS software nuclear translocation wizard was used to determine the similarity coefficient between the NF-κB and DRAQ5 (nuclei) staining's. As described by AM

Silva *et al.* (26), nuclear translocation was considered for NF-κB/DRAQ5 similarity coefficients above 1.

1.2.9 Protein identification by nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS)

Protein identification and quantitation was performed by nano LC-MS/MS. This equipment is composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Germany). Samples were loaded onto a trapping cartridge for 3 min and further separated in a nano-C18 column at 300 nL/min. Data acquisition was controlled by Xcalibur and Tune software (Thermo Scientific). The mass spectrometer was operated in data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from full scan. Raw data was processed using Proteome Discoverer 2.2.0.388 software (Thermo Scientific). Protein identification was performed with Sequest HT search engine against the *Mus musculus* entries from the UniProt database.

1.2.10 Enzyme-linked immunosorbent assay (ELISA)

Supernatants of N9 microglial cells were collected and processed (1500 rpm, 10 min, 4ºC). TNF-α, IL-1β, IL-6, MIP-2, IL-12, IL-10 and IL-4 levels were evaluated by ELISA, according to the manufacturer's instructions (ABTS ELISA Development Kit, PeproTech). Cytokine levels were measured in a plate reader at 405 nm, with wavelength correction at 650 nm. Cytokine concentrations (pg/mL) were determined using a standard calibration curve.

1.2.11 Immunofluorescence

Primary neurons and N9 microglial cells were washed and fixed with 4% PFA in PBS. Cells were permeabilized with 0.25% Triton in PBS prior to blocking and overnight incubation at 4ºC with primary antibodies: mouse anti-β3 Tubulin (Biolegend) and rabbit anti-Iba1 (Wako) for neurons and microglia, respectively. Secondary antibodies anti-mouse Alexa 488 (Cell Signaling Technologies) and anti-rabbit Alexa 594 (Invitrogen) were incubated for 1h at RT. Nuclear staining was performed by incubating cells with Hoechst (Sigma) for 5 min at RT. Coverslips were mounted in microscope slides with Fluoroshield™ (Sigma) and images randomly acquired in a Zeiss Axio Imager Z1 Apotome. Neuronal apoptosis was addressed by evaluating nuclei shape of ten images per condition (27).

1.2.12 Nitrites quantification (Griess assay)

Supernatants from neuron-N9 microglia co-cultures were mixed with an equal volume of Griess reagent in a 96-well plate. Sodium nitrite (1000 nM, Sigma) was serial diluted to

generate the standard curve. Absorbance was read at 550 nm and nitrites concentration calculated using a standard curve.

1.2.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, Inc.). Gaussian distribution was tested by the Shapiro-Wilk normality test. For non-normal distribution data, tests were used to evaluate significant differences between samples, namely Wilcoxon matched-pairs signed rank test (between 2 groups) and Friedman test, followed by uncorrected Dunn's multiple comparison test (more than 2 groups). When the data passed normality tests, one-way ANOVA (more than 2 groups), followed by Sidak's multiple comparison test was used. The statistical test used is identified in each figure legend. Experiments were performed at least 3 times independently. All samples were included in the analysis. Statistical significance was considered for $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: non-significant).

1.3 Results

1.3.1 TNF-α induces microglia activation through NF-κB

To better understand the mechanism by which TNF-α activates microglia, primary rat mixed glial cells (microglia, oligodendrocytes and astrocytes) were isolated from P1-P2 rats. Microglia was obtained using the well described shaking method, which allowed the setup of microglial *in vitro* experiments with a cell purity >99% (Supplementary Figures 1 and 2). To assess the impact of TNF-α on the expression of inflammation-associated genes, microglia was stimulated with 20 ng/mL of recombinant TNF-α for 6h, determined as the peak of NF-κB p65 phosphorylation (Supplementary Figure 3). LPS (100 ng/mL) was used as a positive control of microglia activation. We observed that TNF-α significantly induced the upregulation of *Nos2* [mean fold-change to CTR (FC) = 2.22, $p = 0.027$], *Tnf* (FC = 2.92, $p = 0.014$) and *Il1b* (FC = 2.54, $p = 0.026$) pro-inflammatory mRNAs compared with non-stimulated microglia (Figure 1A). Interestingly, TNF-α had no impact in the expression of *Il6*, *Il10* and *Msr1* mRNAs, whereas LPS induced the upregulation of *Il6* (FC = 4.69, $p = 0.0016$) and *Il10* (FC = 2.28, $p = 0.027$) and downregulation of *Msr1* (FC = 0.19, $p = 0.027$; Figure 1A). Importantly, we also observed that TNF-α-induced microglia activation resulted in increased levels of ph-NF-κB p65 ($p = 0.011$; Figure 1B), suggesting that the overexpression of the inflammatory mRNAs may be achieved through the activation of the NF-κB pathway.

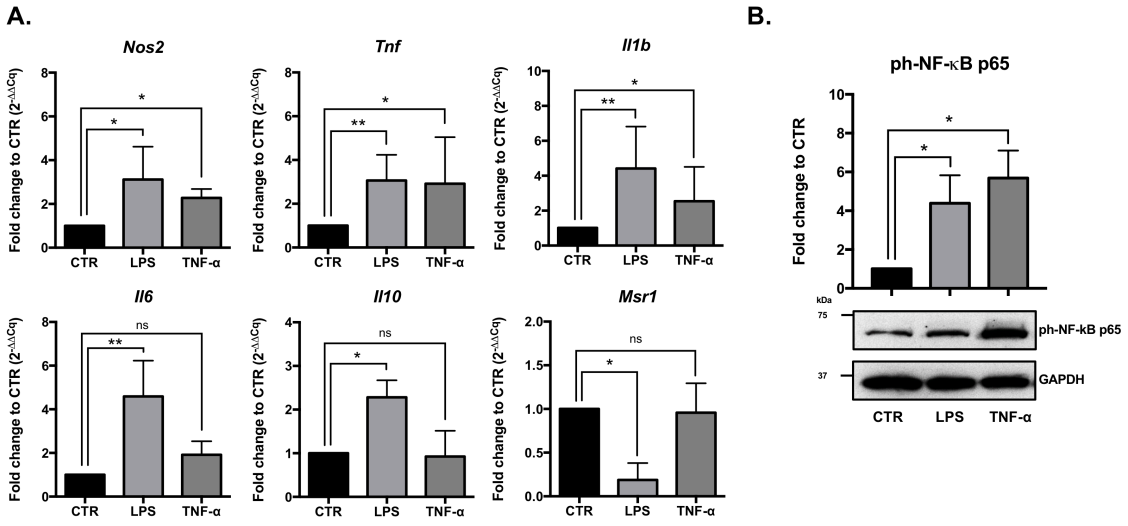


Figure 1. TNF-α induced overexpression of pro-inflammatory genes, through activation of NF-κB pathway in microglia. Rat microglia were obtained from mixed glial cultures. Isolated microglia were re-seeded in 6-well plates, allowed to adhere for 48h and then stimulated for 6h with LPS (100 ng/mL) or TNF-α (20 ng/mL). (A) Gene expression profile of activated microglia evaluated by RT-qPCR. Results were normalized with Gapdh and are expressed in fold change to CTR (mean ± SD, n=5). (B) NF-κB p65 phosphorylation levels evaluation by western blot after microglia activation with LPS or TNF-α (mean ± SD, n=5). GAPDH was used as normalizer. Statistical significance: **p<0.01, * p<0.05, ns - non significant; Friedman test followed by Dunn's multiple comparisons test.

1.3.2 miR-342 is overexpressed in TNF-α activated microglia

In order to explore the role of miRNAs in TNF-α-driven microglia activation, a miRNA microarray was performed in three independent experiments (Figure 2A). Only miRNAs averaging a detection signal > 20 were considered. 19 miRNAs were upregulated (log_2 FC to CTR > 0.2), while 23 were downregulated (log_2 FC to CTR < -0.2) in microglia stimulated with TNF-α compared to control cells (Supplementary Table 3). miR-146b-5p was the most expressed miRNA (FC to CTR = 1.61), however, only miR-342-3p was significantly upregulated (FC to CTR = 1.28, p = 0.03; Figure 2B and Supplementary Table 3). miR-124-3p, the most downregulated miRNA (FC to CTR = -4.15; Figure 2B and Supplementary Table 3), and several members of the let-7 family members were all downregulated on TNF-α activated microglia (Figure 2B), although not statistically different to control. To validate these results, miRNA expression levels were evaluated by RT-qPCR. In fact, microarray results were confirmed, since only miR-342-3p was significantly upregulated after TNF-α stimulation (FC to CTR= 1.39; p=0.0078; n = 8). Neither miR-146b-5p, miR-124-3p, nor let-7i-5p (a member of let-7 family) were significantly altered (Figure 2C). These results suggest that miR-342 may have a role in TNF-α-mediated microglia activation.

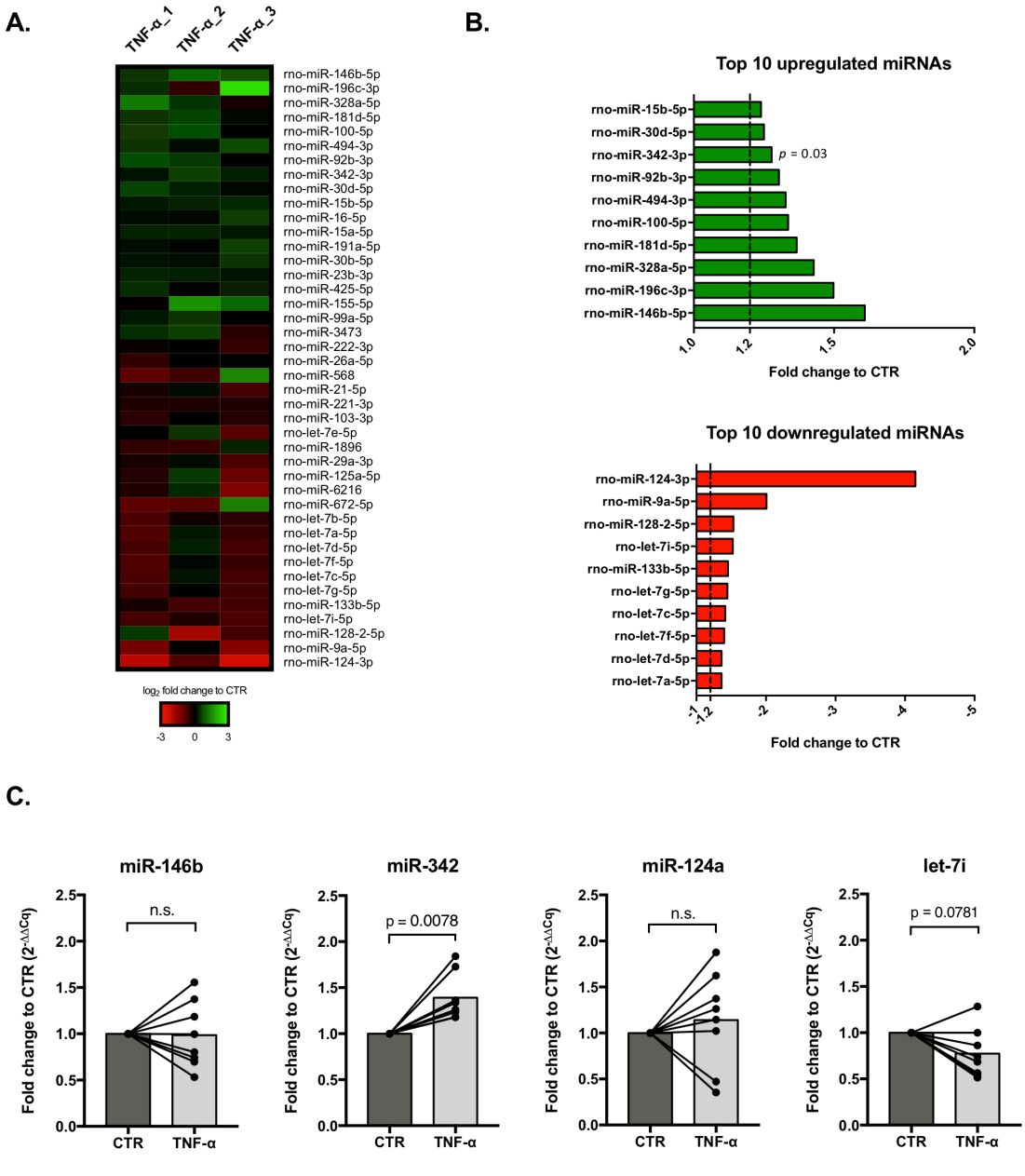


Figure 2. miR-342 is overexpressed in TNF-α stimulated microglia. (A) Heat map of miRNA microarray expression results in TNF-α activated microglia (n=3). Only miRNAs with -0.2 ≤ log2 FC to CTR ≥ 0.2 and a detection signal ≥ 20 are represented. (B) Ten most upregulated (green) or downregulated (red) miRNAs in TNF-α stimulated microglia (mean FC to CTR, n=3). (C) Fold change to control based on the relative expression of the selected miRNAs, evaluated by RT-qPCR (mean, n=8). U6 SnRNA was used as reference. Cq: quantification cycle. Statistical significance: p<0.05; Wilcoxon matched-pairs test.

1.3.3 miR-342 plays a role in TNF-α mediated microglia activation

To investigate the role of miR-342 in microglia activation following TNF-α stimulation, gain-of-function experiments were performed using the N9 microglia cell line (Supplementary Figure 4). Strikingly, we found that miR-342 overexpression *per se*, without TNF-α stimulation,

was sufficient to increase ph-NF-κB-p65 levels compared with non-stimulated (p = 0.004) and SCR-transfected microglia cells (p = 0.012; Figure 3A). On the other hand, anti-miR-342 transfection resulted in a decrease in ph-NF-κB-p65 levels compared with anti-SCR (p = 0.028), but not CTR (Figure 3A). However, following TNF-α activation, miR-342 inhibition had a significant impact on the reduction of NF-κB-p65 phosphorylation levels (*vs* TNF-α, p = 0.044; *vs* TNF-α + anti-SCR, p = 0.037). The translocation of NF-κB to the nucleus under miR-342 overexpression was analysed using ImageStreamX. Figure 3B shows a representative histogram of the similarity coefficient between NF-κB and nuclei of N9 microglia cells transfected with SCR (green) or miR-342 (yellow), and representative images of a cell with NF-κB in the cytoplasm (non-translocated) or co-localized with the nucleus (translocated). Transient transfection of N9 microglia cells with miR-342 resulted in a clear shift towards a higher similarity coefficient, which indicates that compared to SCR, miR-342 overexpression promotes NF-κB translocation to the nucleus. Quantification of the percentage of cells with nuclear localization of NF-κB shows a significant NF-κB nuclear translocation upon miR-342 overexpression (Figure 3B). Although not significantly, the treatment with TNF-α also shows a tendency for inducing the translocation of NF-κB into the nucleus (Figure 3B, 10 min). The inflammatory role of miR-342 was further confirmed by the levels of pro-inflammatory cytokines produced by transfected microglia. Specifically, microglia overexpressing miR-342 show increased levels of TNF-α and IL-1β, compared to non-stimulated (TNF-α, p = 0.021; IL-1β, p = 0.008) and SCR-transfected microglia cells (TNF-α, p = 0.004; IL-1β, p = 0.022; Figure 4). Conversely, miR-342 overexpression had no effect on IL-6, MIP-2, IL-12, IL-10 and IL-4. miR-342 inhibition, without TNF-α stimulation, had no impact on cytokine production compared with anti-SCR control (Figure 4). In agreement with the RT-qPCR results shown in Figure 1A, exposure to TNF-α induced the secretion of TNF-α (p = 0.0006) and IL-1β (p < 0.0001), and also of MIP-2 (p = 0.019) compared with control microglia cells (Figure 4), while no differences were detected for IL-6, IL-12, IL-10 and IL-4 (Figure 4). Importantly, the increased secretion of TNF-α, IL-1β and MIP-2 after TNF-α stimulation, was reduced by miR-342 inhibition (TNF-α, p = 0.004; IL-1β, p = 0.004; MIP-2, p = 0.027, Figure 4), which supports the hypothesis that miR-342 is needed for TNF-α-driven microglia activation.

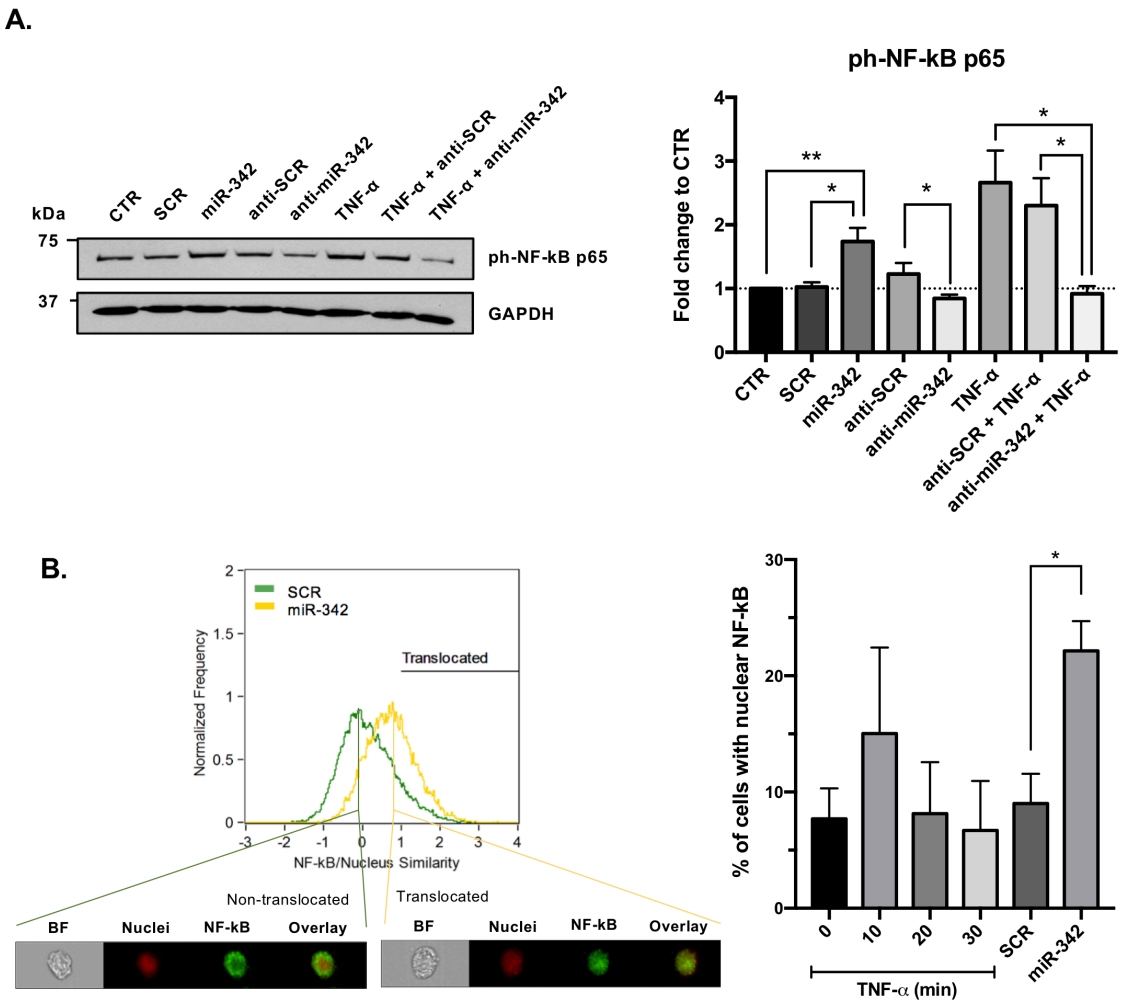


Figure 3. miR-342 regulates TNF-α-mediated microglia activation through NF-κB. (A) ph-NF-κB p65 expression evaluation by western blot after TNF-α stimulation and/or mirVana miRNA mimic/inhibitor mmu-miR-342-3p or mirVana miRNA mimic/inhibitor Negative Control (SCR) transfection. Results were normalized with GAPDH and compared to non-stimulated N9 microglia (mean ± SD, n=3-6). (B) Representative plot of the similarity coefficient between NF-κB and nuclei staining's in cells transfected with mirVana miRNA mimic negative control (SCR, green) or miRNA mimic mmu-miR-342-3p (miR-342, yellow). The black line (Translocated) corresponds to gated cells with nuclear translocated NF-κB (similarity coefficient > 1). Representative images of cells with a similarity coefficient < 1 (non-translocated) and with a similarity coefficient > 1 (translocated) are shown below. BF: brightfield. On the right, graph shows the quantification of the percentage of cells with nuclear translocated NF-κB (translocated gate, similarity coefficient > 1) after exposure to TNF-α for the indicated times or transfection with SCR or miR-342. Results are mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$; ANOVA followed by Sidak's multiple comparison test.

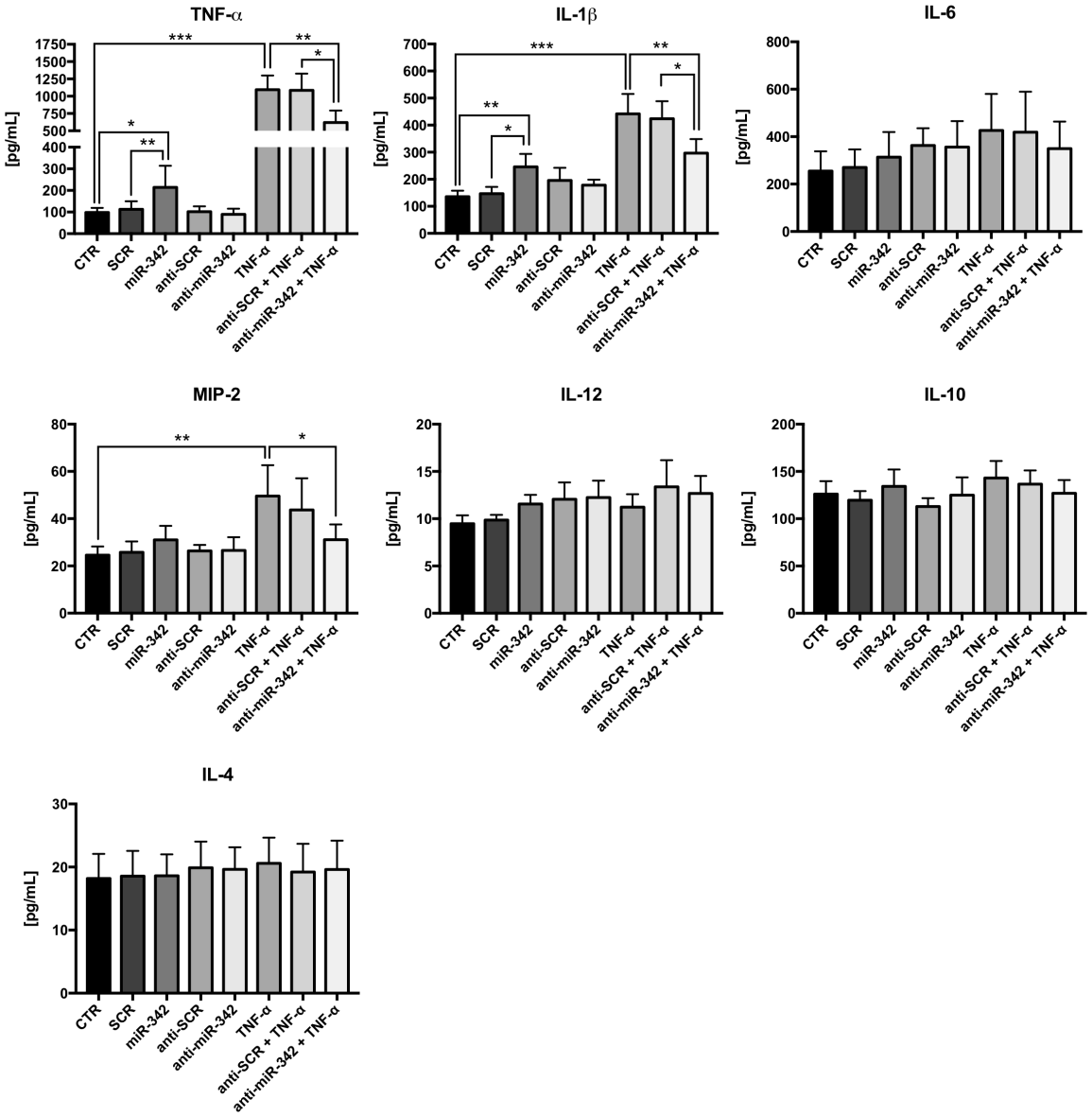
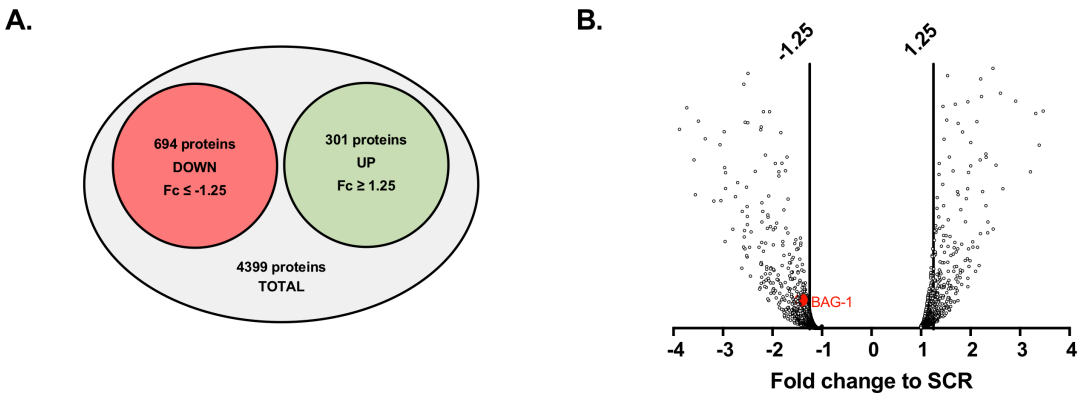


Figure 4. miR-342 impacts cytokine secretion levels in microglia. N9 microglia were transfected with mirVana miRNA mimic/inhibitor mmu-miR-342-3p or mirVana miRNA mimic/inhibitor Negative Control (SCR), using Lipofectamine 2000. When indicated, microglia were also stimulated with TNF-α (20 ng/mL) for 6h. After that, supernatants were collected and stored at -80ºC. Levels of TNF-α, IL-1β, IL-6, MIP-2, IL-12, IL-10 and IL-4 were quantified by ELISA (mean ± SD, n=5-7). Cytokine concentration was obtained using corresponding standard curve, according to manufacturer's instructions. Statistical significance: *p<0.05, **p<0.01 and ***p<0.001; Friedman test followed by Dunn's multiple comparisons test.

1.3.4 miR-342 promotes NF-κB activation by inhibiting BAG-1

Next, we searched for miR-342 target molecules and pathways/functions that could be involved in the TNF-α-driven microglia activation. Thus, the protein expression profile of miR-342 transfected N9 microglia cells was analysed (Figure 5A, B). The nano LC-MS/MS identified 4399 proteins, of which 694 were downregulated (FC to SCR < -1.25) and 301

upregulated (FC > 1.25; Figure 5A, B and Supplementary Table 4). Interestingly, DAVID functional annotation (28), revealed that the group of upregulated proteins with the highest biological function enrichment score, are mainly involved in inflammatory responses (Figure 5C). In the group of most downregulated proteins, we identified a potential candidate, BAG-1 (FC = -1.42; Figure 5B). BAG-1 was previously described to degrade NF-κB p65, inhibiting inflammatory signaling in dendritic cells (29).



C.

Most Upregulated Biological Functions ***(miR-342 vs SCR, FC ≥ 1.25)***

Enrichment Score: 1	*Proteins*	*Count*	*p-value*
Immune system process	CD14, FADD, CR1L, HMGB2, LCN2, MIF, PTMS, POLR3B, POLR3F, PSMB9, TLR13, TAP1	12	$3.3x10^{-2}$
Positive regulation of innate imune response	HMGB2, POLR3B, POLR3F	3	$5.3x10^{-2}$
Innate imune response	CD14, FADD, CR1L, HMGB2, LCN2, MIF, POLR3B, POLR3F, TLR13, RELA	10	$7.9x10^{-2}$
Positive regulation of interferon-beta production	HMGB2, POLR3B, POLR3F	3	$1.1x10^{-1}$
Defense response to virus	POLR3B, POLR3F, FADD	3	$6.9x10^{-1}$

Most Downregulated Biological Functions ***(miR-342 vs SCR, FC ≤ -1.25)***

Enrichment Score: 0.46	*Proteins*	*Count*	*p-value*
Peptidyl-tyrosine phosphorilation	ABI3, JAK1, LYN, CSF1R, INSR	5	$2.3x10^{-2}$
Transmembrane receptor protein tyrosine kinase signaling pathway	JAK1, LYN, INSR	4	$3.2x10^{-2}$
Peptidyl-tyrosine autophosphorylation	JAK1, LYN, CSF1R, INSR	3	$5.7x10^{-2}$

Figure 5. Protein expression profile of miR-342-transfected microglia. Protein identification and quantitation was performed by nano LC-MS/MS. Diagram (A) and volcano plot (B) represent the most differently expressed proteins within all identified proteins (-1.25 ≤ FC to SCR ≥ 1.25, n=2). (C) Most up and downregulated biological functions based on protein expression fold change between miR-342 and SCR. Analysis was performed with DAVID using Functional Annotation Tool. The entire list proteins detected was used as background. “Enrichment score” indicates the biological relevance of the group of proteins involved in the respective set of biological functions, based on the *p*-values of all enriched annotation terms. “Count” indicates the number of dysregulated proteins involved in that specific biological function. Full protein names can be found in Supplementary Table 4.

To validate these results, we carried out western blots against BAG-1 on proteins extracts from miR-342 and anti-miR-342 transfected N9 microglial cells. Results show that miR-342 overexpression significantly inhibits BAG-1 expression (mean FC to SCR = 0.546, $p = 0.031$; Figure 6A), while miR-342 inhibition induces an increase on BAG-1 expression (mean FC to anti-SCR = 1.911, $p = 0.078$; Figure 6A). To confirm if NF-κB activation on miR-342-overexpressing N9 microglia cells occurs via BAG-1 inhibition, we used a siRNA targeting BAG-1 to inhibit its expression. Results clearly show that NF-κB p65 activation is dependent on BAG-1 expression, since its downregulation induced a significant overexpression of ph-NF-κB p65 (mean FC to siRNA NC = 4.18, $p = 0.012$; Figure 6B). To further explore the role of BAG-1 on NF-κB p65 regulation, we transiently transfected N9 microglia cells with a BAG-1 mammalian expression vector. Although not significant, BAG1 overexpression showed a trend towards a slight inhibition of NF-κB p65 phosphorylation levels compared with control (*vs* pCMV6, $p = 0.08$). Nonetheless, it significantly inhibited the upregulation of ph-NF-κB induced by TNF-α (*vs* TNF-α, $p = 0.008$; *vs* TNF-α + pCMV6, $p = 0.014$, Figure 6C). These results show that miR-342 promotes NF-κB activation by inhibiting BAG-1.

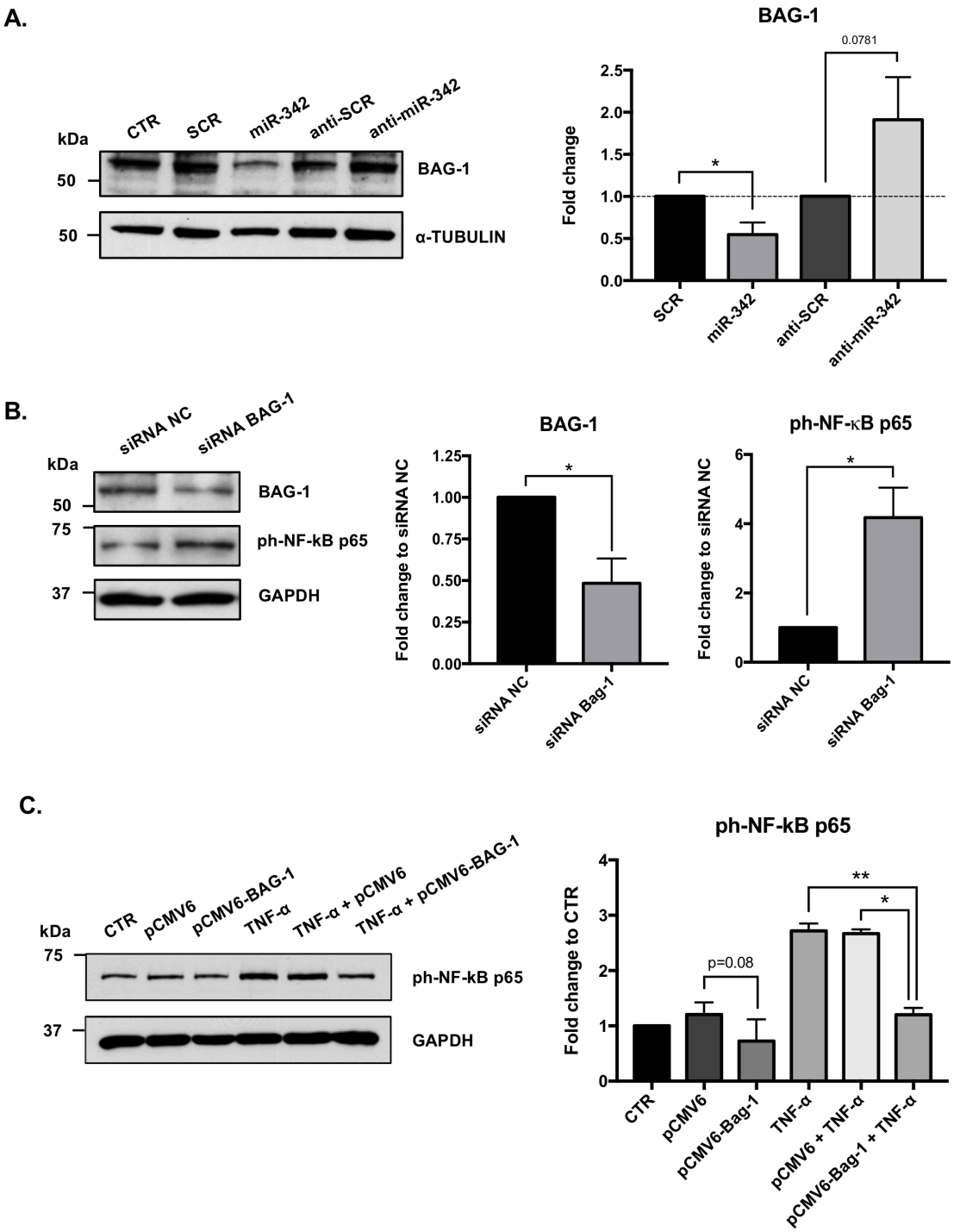


Figure 6. miR-342 induces NF-κB activation by inhibiting BAG-1. (A) BAG-1 expression after miR-342 overexpression/inhibition was addressed by western blot. Results were normalized with α-tubulin and compared to the respective controls (mean ± SD, n=6). To evaluate the involvement of BAG-1 on NF-κB activation, N9 microglia were transfected with a siRNA to silence (B) or with a plasmid (1ug/mL) to overexpress BAG-1 (C). BAG-1 and ph-NF-κB p65 expression levels were evaluated by western blot. Results were normalized with GAPDH and compared to the respective controls (mean ± SD, n=2-4). Statistical significance: *p<0.05, Wilcoxon matched-pairs test.

1.3.5 miR-342 overexpression in microglia induces neurotoxicity

After identifying the mechanism by which miR-342 participates on microglia activation, we addressed its functional consequences, specifically in the crosstalk with neurons. Primary mouse hippocampal neurons were cultured on the right side of microfluidic chambers for 13 days, allowing axon projection through the device's microgrooves. Then, transfected/TNF-α stimulated N9 microglia cells were plated on the left side of the system (Figure 7A and Supplementary Figure 5). After 24h of co-culture, the supernatants were collected, and the cells were immunostained for the assessment of neuronal viability. Interestingly, TNF-α activated and miR-342 transfected microglia had a significant impact on neurons integrity (Figure 7B). TNF-α stimulated microglia significantly reduced neuron viability by 55%, compared with non-stimulated microglia (FC to CTR = 0.45, p = 0.028; Figure 7C). miR-342-overexpressing N9 microglia cells also significantly induced neurotoxicity, reducing cell viability by 33% compared with CTR (FC to CTR = 0.67, p = 0.041, Figure 7C) and in 38% compared with SCR-transfected microglia (FC to CTR = 0.62, p = 0.026, Figure 7C).

Considering that overactivated microglia increases the production of pro-inflammatory cytokines and nitric oxide species, which may have deleterious effect to the surrounding cells, we next determined nitrites concentration in the co-culture supernatants. In fact, the quantification shows a significant increase in the levels of nitrites in the supernatants of both TNF-α-stimulated (p = 0.0437) and miR-342-overexpressing (p = 0.0437) microglia co-cultures (Figure 7D). These results support our hypothesis that miR-342-mediated microglia activation impacts the crosstalk with neurons.

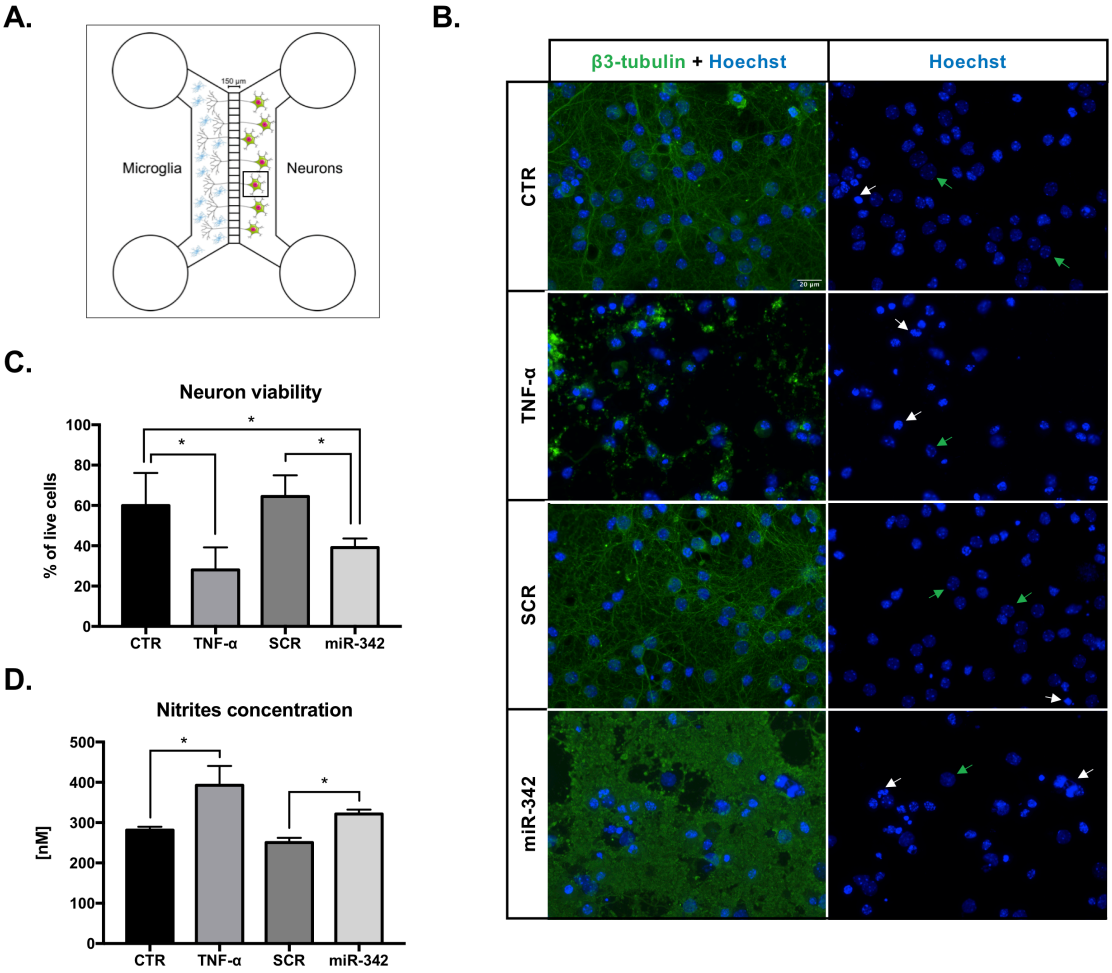


Figure 7. miR-342 overexpression in microglia induces neurotoxicity. (A) Neurons were cultured in PDL-coated coverslips previously attached to the Axon Investigation System. At day 13 of neuronal culture, transfected, TNF-α or non-stimulated N9 microglia were added to the respective system, in direct contact with axons for 24h. (B) Immufluorescence images of neurons after co-culture with N9 microglia. Left panel shows neurons stained with anti-β3-tubulin (anti-Alexa 488, green) and Hoechst (blue). Right panel shows only Hoechst nuclear staining used for neuron viability evaluation. White arrows highlight the nucleus of dead neurons, while green arrows highlight the nucleus of healthy neurons. Scale bar: 20 µm. (C) Neuron viability was addressed after counting the number of living and dead cells of 10 images per condition (mean ± SD, n=4). (D) Co-cultures' supernatants were collected for nitrite levels quantification using Griess reagent (mean ± SD, n=4). Statistical significance: * $p<0.05$ and ** $p<0.01$, Friedman test followed by Dunn's multiple comparisons test.

1.4 Discussion

Microglia are the primary immune effector cells in the CNS. Impairment of their normal structure or function, caused by either inflammatory activation (e.g., following infection, trauma, autoimmune or neurodegenerative diseases) or by their decline and senescence (e.g., during aging or Alzheimer's disease), may cause damage in neuronal function and neurogenesis (30-33). Additionally, systemic alterations in the cytokine profile have been associated with distinct neurological and psychiatric diseases (e.g., depression, bipolar disorder or schizophrenia) (30, 34, 35). Pro-inflammatory cytokines, such as TNF-α, a central mediator of neuroinflammation (36), have access to the brain through humoral and neural routes, leading to microglia activation (37). In this work, we explored the impact of TNF-α on miRNAs expression in microglia and the mechanisms by which these miRNAs regulate cell activation. We show, for the first time, that miR-342 is a crucial mediator of TNF-α-driven microglia activation.

miR-342 was found upregulated in primary rat microglia activated with TNF-α. To explore the role of miR-342 on TNF-α-driven microglia activation we used a mouse cell line (N9), due to the low (non-viral) transfection efficiency when working with primary microglia cells. Moreover, of the few microglia cell lines available, N9 is the one of the most commonly used and widely accepted. Importantly, the miR-342-3p sequence shows 100% homology between rat and mouse (Supplementary Figure 6).

Following miR-342 overexpression, we found that miR-342 *per se*, was sufficient to activate the NF-κB pathway, as shown by the increased ph-NF-κB p65 levels and NF-κB p65 nuclei translocation. Notably, miR-342-driven NF-κB p65 activation led to increased secretion of TNF-α and IL-1β. Conversely, miR-342 inhibition strongly decreased the levels of these cytokines after TNF-α stimulation, suggesting that this miRNA is needed for microglia activation. Moreover, we observed that TNF-α mRNA and secreted levels were upregulated after TNF-α stimulation, in a positive feedback loop, possibly perpetuating neuroinflammation and ultimately promoting brain pathological conditions (13). In line with our results, Kuno *et al*. proposed the microglia-derived TNF-α autocrine activation occurs via TNFR1 signaling pathway (13).

miRNAs act by degradation or translation inhibition of their target mRNAs (16). Therefore, after observing that miR-342 overexpression increases the levels of ph-NF-κB p65, we hypothesized that this miRNA could be repressing an inhibitor of NF-κB. Consequently, we screened the LC-MS/MS data for proteins that were significantly downregulated and previously described as repressors of NF-κB. BAG-1 was the only candidate found to meet both criteria (29) (Supplementary Table 4). BAG-1 is known to interact with heat shock protein 70 (Hsp70) family of molecular chaperones, displaying a variety of cytoprotective activities

and effects on signal transduction and transcription, suggesting a function in overcoming cellular stress and inflammation (38, 39). Tanaka *et al.* described that BAG-1, when associated with HSP70, can promote NF-κB p65 degradation in LPS-treated dendritic cells (29). The authors reported that mouse dendritic cells deficient in either HSP70 or BAG-1 had more nuclear p65 and produced more proinflammatory cytokines than did wild-type dendritic cells (29), implicating BAG-1 as a negative regulator of pro-inflammatory NF-κB signalling in dendritic cells. Our study shows, for the first time, that BAG-1 is a mediator of NF-κB signalling in microglia, and that BAG-1 protein levels are regulated by miR-342.

Nevertheless, it is important to note that miRNAs can target multiple transcripts, as such we do not exclude that other proteins with relevance for microglia activation, can also be affected by miR-342 overexpression. Specifically, DAVID functional annotation analysis revealed that inflammation-related processes were the most upregulated biological functions associated with miR-342 overexpression (Figure 5C). Particularly, FADD and MIF expression regulation by miR-342 should be further explored, as they are known to potentiate NF-κB activation and pro-inflammatory cytokines production (40, 41).

The deleterious effects of excessive production of cytokines and nitric oxide species in neurons have been extensively studied (32, 37). For instance, TNF-α can potentiate glutamate-mediated cytotoxicity by two complementary mechanisms: indirectly, by inhibiting glutamate transport on astrocytes, and directly, by stimulating extensive microglial and/or astrocyte glutamate release in an autocrine manner. This disturbs the balance of excitation and inhibition, resulting in a higher synaptic excitatory/inhibitory ratio – excitoneurotoxicity (37, 42, 43). On the other hand, microglial derived nitric oxide inhibits neuronal respiration resulting in glutamate release and subsequent excitotoxicity (44, 45). As such, to address the effect of miR-342 overexpression in the crosstalk with neurons, we co-cultured microglia with hippocampal neurons. Interestingly, we found that, in both TNF-α stimulated and miR-342 transfected microglia, neuron viability was drastically affected compared with the controls. We hypothesize that this may be due to an increased production of the pro-inflammatory cytokines IL-1β and TNF-α (Results section 3), but also due to increased levels of nitrites detected in the supernatants of these co-cultures. Not surprisingly, and given the upregulation of *Nos2*, supernatants of TNF-α stimulated microglia show an increased level of nitrites. Moreover, miR-342 overexpression also induced neuronal cell death, revealing that miR-342 impacts microglia activation as well as its crosstalk with neurons. In the current work the possibility that these findings are cell line specific cannot be completely ruled out. It would be interesting to pursue this work in primary microglia cells, and further to perform *in vivo* administration of miR-342 targeted to microglia, to clarify its impact on the crosstalk between microglia and neurons, as well as the repercussion on animals' behaviour, particularly in animal models of neuroinflammation.

These findings support miR-342 as potential target to resolve neuroinflammation, characterized by increased levels of TNF-α, sustained microglia activation, and often associated with the development of neurological and psychiatric disorders.

Acknowledgments

Authors would like to thank Dr. João Relvas lab for the help with N9 microglia cell culture; Dr. Sofia Lamas for the guidance on the animal welfare and support with animal experiments (Animal facility, i3S); and to LC Sciences for the miRNA microarray data and analysis. The mass spectrometry technique was performed by Hugo Osório at the i3S Proteomics Scientific Platform with support from the Portuguese Mass Spectrometry Network, integrated in the National Roadmap of Research Infrastructures of Strategic Relevance (ROTEIRO/0028/2013; LISBOA-01-0145-FEDER-022125). This work was funded by project NORTE-01-0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). JPB and JB are supported by FCT- Fundação para a Ciência e Tecnologia, through BiotechHealth PhD program fellowship (PD/BD/135490/2018) and Areas of Basic and Applied Biology PhD program fellowship (PD/BD/135450/2017), respectively.

References

1. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nat Rev Immunol. 2016;16(1):22-34.
2. Yirmiya R, Rimmerman N, Reshef R. Depression as a microglial disease. Trends in neurosciences. 2015;38(10):637-58.
3. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science (New York, NY). 2010;330(6005):841-5.
4. Thion MS, Ginhoux F, Garel S. Microglia and early brain development: An intimate journey. Science. 2018;362(6411):185-9.
5. Ransohoff RM, Perry VH. Microglial physiology: unique stimuli, specialized responses. Annual review of immunology. 2009;27:119-45.
6. Dheen ST, Kaur C, Ling EA. Microglial activation and its implications in the brain diseases. Current medicinal chemistry. 2007;14(11):1189-97.
7. Perry VH, Holmes C. Microglial priming in neurodegenerative disease. Nat Rev Neurol. 2014;10(4):217-24.

8. Welser-Alves JV, Milner R. Microglia are the major source of TNF-α and TGF-β1 in postnatal glial cultures; regulation by cytokines, lipopolysaccharide, and vitronectin. Neurochem Int. 2013;63(1):47-53.

9. Riazi K, Galic MA, Kuzmiski JB, Ho W, Sharkey KA, Pittman QJ. Microglial activation and TNFα production mediate altered CNS excitability following peripheral inflammation. Proceedings of the National Academy of Sciences. 2008;105(44):17151.

10. Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future. Cytokine & Growth Factor Reviews. 2014;25(4):453-72.

11. Golan H, Levav T, Mendelsohn A, Huleihel M. Involvement of Tumor Necrosis Factor Alpha in Hippocampal Development and Function. Cerebral Cortex. 2004;14(1):97-105.

12. Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nature reviews Neuroscience. 2007;8.

13. Kuno R, Wang J, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. Autocrine activation of microglia by tumor necrosis factor-alpha. J Neuroimmunol. 2005;162(1-2):89-96.

14. Kolshus E, Dalton VS, Ryan KM, McLoughlin DM. When less is more--microRNAs and psychiatric disorders. Acta psychiatrica Scandinavica. 2014;129(4):241-56.

15. Geaghan M, Cairns MJ. MicroRNA and Posttranscriptional Dysregulation in Psychiatry. Biological psychiatry. 2015;78(4):231-9.

16. Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutation research. 2011;717(1-2):1-8.

17. Almeida MI, Reis RM, Calin GA. Decoy activity through microRNAs: the therapeutic implications. Expert opinion on biological therapy. 2012;12(9):1153-9.

18. Almeida MI, Nicoloso MS, Zeng L, Ivan C, Spizzo R, Gafa R, et al. Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. Gastroenterology. 2012;142(4):886-96.

19. Almeida MI, Calin GA. The miR-143/miR-145 cluster and the tumor microenvironment: unexpected roles. Genome Medicine. 2016;8:29.

20. Bras JP, Silva AM, Calin GA, Barbosa MA, Santos SG, Almeida MI. miR-195 inhibits macrophages pro-inflammatory profile and impacts the crosstalk with smooth muscle cells. PloS one. 2017;12(11):e0188530.

21. Alexander M, Hu R, Runtsch MC, Kagele DA, Mosbruger TL, Tolmachova T, et al. Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. Nature communications. 2015;6:7321.

22. Tamashiro TT, Dalgard CL, Byrnes KR. Primary microglia isolation from mixed glial cell cultures of neonatal rat brain tissue. Journal of visualized experiments : JoVE. 2012(66):e3814.

23. Seibenhener ML, Wooten MW. Isolation and culture of hippocampal neurons from prenatal mice. Journal of visualized experiments : JoVE. 2012(65):3634.

24. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.

25. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. Nucleic Acids Res. 2019;47(D1):D155-d62.

26. Silva AM, Oliveira MI, Sette L, Almeida CR, Oliveira MJ, Barbosa MA, et al. Resveratrol as a natural anti-tumor necrosis factor-alpha molecule: implications to dendritic cells and their crosstalk with mesenchymal stromal cells. PloS one. 2014;9(3):e91406.

27. Crowley LC, Marfell BJ, Waterhouse NJ. Analyzing Cell Death by Nuclear Staining with Hoechst 33342. Cold Spring Harbor protocols. 2016;2016(9).

28. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009;4(1):44-57.

29. Tanaka T, Shibazaki A, Ono R, Kaisho T. HSP70 mediates degradation of the p65 subunit of nuclear factor kappaB to inhibit inflammatory signaling. Science signaling. 2014;7(356):ra119.

30. Miller AH, Haroon E, Raison CL, Felger JC. Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. Depression and anxiety. 2013;30(4):297-306.

31. Zhang L, Zhang J, You Z. Switching of the Microglial Activation Phenotype Is a Possible Treatment for Depression Disorder. Frontiers in cellular neuroscience. 2018;12:306-.

32. Brown GC, Neher JJ. Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. Mol Neurobiol. 2010;41.

33. Streit WJ, Mrak RE, Griffin WST. Microglia and neuroinflammation: a pathological perspective. Journal of neuroinflammation. 2004;1(1):14-.

34. Stuart MJ, Baune BT. Chemokines and chemokine receptors in mood disorders, schizophrenia, and cognitive impairment: a systematic review of biomarker studies. Neuroscience and biobehavioral reviews. 2014;42:93-115.

35. Rosenblat JD, Cha DS, Mansur RB, McIntyre RS. Inflamed moods: a review of the interactions between inflammation and mood disorders. Progress in neuro-psychopharmacology & biological psychiatry. 2014;53:23-34.

36. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol. 2006;27(1):24-31.

37. Olmos G, Llado J. Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. Mediators of inflammation. 2014;2014:12.

38. Gotz R, Wiese S, Takayama S, Camarero GC, Rossoll W, Schweizer U, et al. Bag1 is essential for differentiation and survival of hematopoietic and neuronal cells. Nat Neurosci. 2005;8(9):1169-78.

39. Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, et al. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell. 1995;80(2):279-84.

40. Kreuz S, Siegmund D, Rumpf J-J, Samel D, Leverkus M, Janssen O, et al. NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. J Cell Biol. 2004;166(3):369-80.

41. Zhang Y, Gu R, Jia J, Hou T, Zheng LT, Zhen X. Inhibition of macrophage migration inhibitory factor (MIF) tautomerase activity suppresses microglia-mediated inflammatory responses. Clinical and Experimental Pharmacology and Physiology. 2016;43(11):1134-44.

42. Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, et al. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. The Journal of biological chemistry. 2006;281(30):21362-8.

43. Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, et al. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci. 2001;4(7):702-10.

44. Boje KM, Arora PK. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. Brain Res. 1992;587(2):250-6.

45. Bal-Price A, Brown GC. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2001;21(17):6480-91.

Supplementary information

TNF-α-induced microglia activation requires miR-342: impact on NF-κB signaling and neurotoxicity

João P. Brás, Joana Bravo, Jaime Freitas, Mário A. Barbosa, Susana G. Santos, Teresa Summavielle, Maria I. Almeida. **TNF-alpha-induced microglia activation requires miR-342: impact on NF-κB signalling and neurotoxicity.** Cell Death Dis. 2020 Jun 2;11(6):415. doi: 10.1038/s41419-020-2626-6.

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Supplementary Table 1

Supplementary Table 1. Oligonucleotides sequences for RT-qPCR. Oligonucleotides used to amplify rat mRNAs encoding inflammatory markers and rat Gapdh used as a reference gene, based on GenBank sequences. Abbreviations: Nos2, nitric oxide synthase 2; Il6, interleukin 6; Tnf, tumour necrosis factor; Il1b, interleukin 1 beta; Msr1, macrophage scavenger receptor 1; Il10, Interleukin 10 macrophage; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

Gene	Accession Number	Forward (5'-3')	Reverse (5'-3')
Rat *Nos2*	NM_012611	AACTTGAGTGAGGAGCAGGTTGA	CGCACCGAAGATATCCTCATGA
Rat *Il6*	NM_012589	CATATGTTCTCAGGGAGATCTTGGA	CAGTGCATCATCGCTGTTCA
Rat *Tnf*	NM_012675	CCCAGACCCTCACACTCAGAT	TTGTCCCTTGAAGAGAACCTG
Rat *Il1b*	NM_031512	CACCTTCTTTTCCTTCATCTTTG	GTCGTTGCTTGTCTCTCCTTGTA
Rat *Msr1*	NM_001191939	AAAGGCAGGGAGGTCAGGATTTC	CCGCTACCATCAACCAGTCG
Rat *Il10*	NM_012854	CATATGTTCTCAGGGAGATCTTGGA	AAACTCATTCATGGCCTTGTA
Rat *Gapdh*	NM_017008	CCCCCAATGTATCCGTTGTG	TAGCCCAGGATGCCCTTTAGT

Supplementary Table 2

Supplementary Table 2. Antibodies used for protein detection. Antibodies were diluted following manufacturer's instructions in 5% BSA in TBS-T 0.1% or 5% Milk in TBS-T 0.1%, for detection of phosphorylated or total forms, respectively. Abbreviations: NF-κB p65, nuclear factor kappa B subunit p65; BAG-1, BAG family molecular chaperone regulator 1; COX-2, cyclooxygenase-2.

Antibody	Molecular Weight (kDa)	Dilution	Manufacturer
Phospho-NF-κB p65	65	1:1000	Cell Signaling
BAG-1	52	1:200	Santa Cruz Biotechnology
COX-2	72	1:200	Santa Cruz Biotechnology
α-TUBULIN	50	1:1000	Cell Signaling
GAPDH	37	1:1000	Cell Signaling
Anti-mouse IgG, HRP-linked	-	1:10000	GE Healthcare
Anti-rabbit IgG, HRP-linked	-	1:10000	GE Healthcare

Supplementary Table 3

Supplementary Table 3. miRNA microarray results. Most up- and downregulated miRNAs in TNF-α versus non-stimulated (CTR) primary rat microglia. Cy3 signal values are in arbitrary units (AU).

Reporter Name	S01 CTR_1-cy3 Signal	S02 CTR_2-cy3 Signal	S03 CTR_3-cy3 Signal	S04 TNF_1-cy3 Signal	S05 TNF_2-cy3 Signal	S06 TNF_3-cy3 Signal	Mean CTR	Mean TNF	log2 (TNF1 / CTR1)	log2 (TNF2 / CTR2)	log2 (TNF3 / CTR3)	Log2 (TNF / CTR)	*p*-value
rno-miR-146b-5p	323	91	152	457	190	265	189	304	0.501	1.054	0.801	**0.69**	**0.338**
rno-miR-196c-3p	55	147	26	75	106	162	76	114	0.444	-0.479	2.622	**0.58**	**0.448**
rno-miR-328a-5p	80	111	131	194	154	112	108	153	1.273	0.469	-0.225	**0.51**	**0.190**
rno-miR-181d-5p	74	86	70	104	134	75	77	105	0.505	0.647	0.099	**0.45**	**0.236**
rno-miR-100-5p	253	116	264	377	197	272	211	282	0.575	0.765	0.043	**0.42**	**0.373**
rno-miR-494-3p	332	553	270	466	618	450	385	511	0.486	0.162	0.736	**0.41**	**0.292**
rno-miR-92b-3p	47	76	94	79	111	92	72	94	0.754	0.555	-0.029	**0.38**	**0.266**
rno-miR-342-3p	**856**	**686**	**753**	**989**	**1,031**	**911**	**765**	**977**	**0.208**	**0.588**	**0.276**	**0.35**	**0.030 ***
rno-miR-30d-5p	262	469	393	408	575	421	374	468	0.641	0.295	0.101	**0.32**	**0.311**
rno-miR-15b-5p	360	593	394	426	738	507	449	557	0.244	0.314	0.363	**0.31**	**0.417**
rno-miR-16-5p	1,144	804	979	1,254	872	1,501	976	1,209	0.133	0.116	0.617	**0.31**	**0.342**
rno-miR-15a-5p	179	111	139	222	141	162	143	175	0.311	0.339	0.213	**0.29**	**0.371**
rno-miR-191a-5p	2,541	1,988	2,056	2,787	2,109	3,125	2,195	2,674	0.133	0.085	0.605	**0.28**	**0.254**
rno-miR-30b-5p	617	693	767	701	762	1,066	692	843	0.186	0.135	0.474	**0.28**	**0.315**
rno-miR-23b-3p	654	519	814	814	633	925	662	791	0.316	0.286	0.185	**0.26**	**0.346**
rno-miR-425-5p	144	176	171	197	175	210	164	194	0.451	-0.007	0.300	**0.25**	**0.098**
rno-miR-155-5p	470	24	69	435	72	142	188	217	-0.111	1.564	1.042	**0.20**	**0.882**
rno-miR-99a-5p	468	235	439	554	327	436	381	439	0.244	0.474	-0.012	**0.20**	**0.587**
rno-miR-3473	254	343	457	342	518	350	351	404	0.428	0.594	-0.382	**0.20**	**0.559**
rno-miR-222-3p	212	68	78	191	67	54	119	104	-0.151	-0.019	-0.529	**-0.20**	**0.822**
rno-miR-26a-5p	857	442	785	620	415	752	695	596	-0.466	-0.091	-0.062	**-0.22**	**0.575**
rno-miR-568	194	429	75	103	286	201	233	197	-0.911	-0.583	1.422	**-0.24**	**0.779**
rno-miR-21-5p	569	282	426	489	316	277	426	360	-0.219	0.164	-0.625	**-0.24**	**0.571**
rno-miR-221-3p	253	69	102	210	58	85	141	118	-0.271	-0.251	-0.268	**-0.27**	**0.762**
rno-miR-103-3p	248	141	199	190	137	158	196	162	-0.390	-0.035	-0.332	**-0.28**	**0.397**
rno-let-7e-5p	79	34	118	74	48	66	77	63	-0.089	0.478	-0.831	**-0.30**	**0.621**
rno-miR-1896	403	650	222	295	448	280	425	341	-0.452	-0.536	0.335	**-0.32**	**0.582**
rno-miR-29a-3p	210	110	215	176	121	130	178	142	-0.249	0.137	-0.729	**-0.32**	**0.419**
rno-miR-125a-5p	52	114	245	41	165	119	137	108	-0.348	0.536	-1.045	**-0.34**	**0.696**
rno-miR-6216	23,627	14,419	24,313	19,700	18,500	9,593	20,787	15,931	-0.262	0.360	-1.342	**-0.38**	**0.342**
rno-miR-672-5p	120	274	46	65	152	117	147	111	-0.883	-0.848	1.344	**-0.40**	**0.662**
rno-let-7b-5p	155	169	211	95	146	158	178	133	-0.700	-0.214	-0.422	**-0.42**	**0.151**
rno-let-7a-5p	405	177	423	239	205	294	335	246	-0.759	0.214	-0.526	**-0.44**	**0.380**
rno-let-7d-5p	329	147	365	207	176	235	281	206	-0.668	0.252	-0.632	**-0.44**	**0.385**
rno-let-7f-5p	457	164	396	273	176	278	339	242	-0.744	0.103	-0.511	**-0.48**	**0.397**
rno-let-7c-5p	281	132	324	168	148	204	246	174	-0.736	0.167	-0.666	**-0.50**	**0.342**
rno-let-7g-5p	167	49	155	106	48	102	124	85	-0.650	-0.037	-0.595	**-0.53**	**0.433**
rno-miR-133b-5p	2,900	8,050	3,366	2,457	5,209	2,169	4,772	3,278	-0.239	-0.628	-0.634	**-0.54**	**0.487**
rno-let-7i-5p	118	42	98	76	34	59	86	56	-0.637	-0.299	-0.725	**-0.61**	**0.335**
rno-miR-128-2-5p	223	470	268	321	136	170	320	209	0.527	-1.788	-0.656	**-0.62**	**0.311**
rno-miR-9a-5p	42	37	119	19	34	46	66	33	-1.195	-0.098	-1.377	**-1.01**	**0.337**
rno-miR-124-3p	148	14	83	35	8	16	81	20	-2.087	-0.757	-2.373	**-2.05**	**0.249**

Supplementary Table 4

Supplementary Table 4. Most up- and downregulated proteins in miR-342 *versus* SCR transfected N9 microglia. Protein identification and quantitation was performed by nano LC-MS/MS. List contains the most up (FC > 1.25, blue) and downregulated (FC < 0.8/-1.25, green) proteins based on expression fold change between miR-342 and SCR (adjusted *p*-value < 0.05).

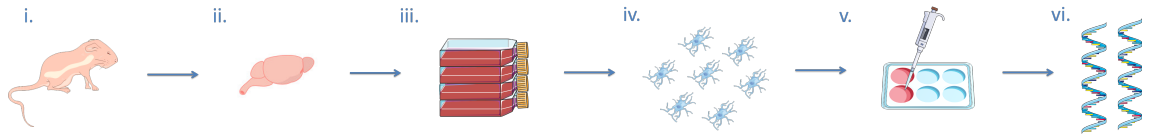
Protein ID	Abundance Ratio	Abundance Ratio Adj. P-Value	Abundances (Grouped)	Abundances (Grouped)	Protein FDR Confidence
	miR-342/SCR	miR-342/SCR	miR-342	SCR	Combined
UPRT	23.238	5.677E-16	4647.5	200	High
TAF12	22.025	5.677E-16	4405	200	High
CYP4A14	12.846	5.677E-16	2569.1	200	Low
ENDOG	11.472	5.677E-16	219.7	19.1	High
EXTL2	10.029	1.8015E-12	2005.7	200	Medium
SRD5A3	8.975	5.677E-16	1795	200	High
PIK3CB	8.611	5.677E-16	1722.2	200	High
MCUR1	8.543	1.9776E-11	1708.6	200	High
PTMS	8.153	2.0068E-10	1630.6	200	High
CST6	7.042	1.0481E-13	1408.4	200	Low
DENND6A	6.939	1.8955E-09	1387.9	200	High
RAPGEF4	6.785	9.3413E-14	191.1	28.2	High
K16	6.552	7.1759E-14	889.3	75.7	High
PSMB9	6.512	5.677E-16	502.6	65	High
DDT	6.149	1.1274E-11	306.7	49.9	High
SSUH2	5.267	7.7522E-09	1053.4	200	Low
PRC1	4.746	1.6097E-10	949.2	200	High
CYLD	4.692	3.8631E-07	938.4	200	High
MCRIP1	4.635	1.3735E-06	430.8	92.9	High
UBXN6	4.453	5.677E-16	1503.2	94.4	High
AAGAB	4.356	1.4922E-11	871.1	200	High
CCDC12	4.338	5.677E-16	258	59.5	High
ARF5	4.276	8.0242E-07	388.9	90.9	High
MAP4	4.268	2.0835E-08	853.5	200	Low
LAMC1	4.227	1.0983E-06	845.5	200	Medium
TSR3	4.195	1.8125E-09	179.1	42.7	High
EEF1D	3.855	1.196E-07	770.9	200	High
TSC1	3.546	1.4424E-06	709.3	200	High
TMCO3	3.469	7.7416E-05	209.5	60.4	High
AASDHPPT	3.388	0.00034216	183.5	54.2	High
KRT42	3.384	6.8553E-08	285.4	84.3	High
INO80	3.316	8.637E-05	663.1	200	Medium
AKR1B3	3.26	1.9376E-06	652	200	High
MTIF2	3.212	0.00109429	642.5	200	High
TAF15	2.911	5.0241E-05	216.3	74.3	High
SMG9	2.655	2.5512E-06	137.6	51.8	High
IST1	2.653	0.00228357	43.3	93.8	High
TSC22D2	2.617	3.0137E-08	237.1	90.6	High
DBR1	2.606	3.5712E-05	75.3	95.8	High
PPT2	2.516	0.00082386	167.9	66.7	High
MINDY3	2.478	3.5852E-06	495.6	200	High
BCAS3	2.457	0.01327979	491.4	200	High
ITIH3	2.452	1.2147E-05	241.8	92.1	High
BCLAF1	2.426	3.3367E-10	305.4	200	High
TMEM177	2.383	9.7446E-07	476.7	200	High
CNOT8	2.353	0.00972524	197.2	83.8	High
BRD8	2.345	0.01565076	469	200	High
ATOX1	2.318	0.00058292	463.7	200	High
H2AW	2.316	5.677E-16	243.4	99.4	High
GDAP2	2.313	0.00050354	231.2	100	High
CETN2	2.294	1.9055E-09	229	72.5	High
LRWD1	2.264	6.5073E-07	222.1	98.1	High
CR1L	2.246	0.0026032	449.2	200	High
DDX55	2.222	4.1026E-05	62.3	200	High
MYC	2.203	1.9194E-05	135.3	61.4	Low
ITPR2	2.201	0.01620303	200	200	High
COX1	2.197	0.00065402	424.8	96.6	High
LACC1	2.151	6.9844E-06	430.2	200	High

POGLUT3	2.142	0.02943154	176.2	82.2	High
LTV1	2.127	0.00012275	211.6	45.2	High
SYNE1	2.09	6.9598E-06	185.1	88.6	Low
CRELD1	2.05	0.02926357	410	200	High
CRADD	2.044	0.02325839	162.1	79.3	High
LEMD2	2.003	0.00030554	266.3	85.5	High
SETD7	2.003	0.00084851	148.7	74.2	High
LHPP	1.991	1.3495E-06	257	99.2	High
KRT1	1.979	2.4515E-12	165.3	97.1	High
MRPL30	1.958	0.03133431	160	81.7	High
CERS6	1.947	5.2039E-05	194.7	100	High
ACTR5	1.943	0.00229041	143.6	99.2	High
TULP3	1.939	0.04855559	139.3	71.8	High
KCTD5	1.914	0.00351456	97.2	50.8	High
MAPKAP1	1.896	0.00963082	188.3	99.3	Low
HIST2H2AB	1.861	9.4659E-09	244	97.3	High
ZNF207	1.841	0.0001935	154.9	76.8	High
ANKS1	1.821	0.02580484	196.1	58.1	High
FBXW11	1.806	0.00572234	136.6	75.6	High
KRT78	1.804	1.9488E-10	165	99.4	High
H3F3C	1.749	0.00013298	173.3	99	High
PEX1	1.742	0.03159714	124	71.2	High
TMTC3	1.739	0.00260124	347.7	200	High
CNOT10	1.71	0.03616002	95.1	55.6	High
NUDCD3	1.695	5.7705E-05	211.8	99.7	High
INTS9	1.684	0.02390757	168.1	99.8	High
HMGB2	1.678	2.711E-08	169.4	99.4	High
PIEZO1	1.671	0.02989171	334.2	200	High
TRMU	1.654	0.03946552	89.8	97.6	High
APPL1	1.649	0.00105168	156.2	97.7	High
KRT73	1.636	8.5408E-08	153.2	93.6	High
FTH1	1.62	4.0934E-06	149	92.2	High
KRT14	1.603	0.04201333	80.5	77.7	High
MIF	1.597	7.9902E-06	158.5	99.2	High
ERLIN1	1.587	0.01843383	148	97.6	High
KCNAB2	1.575	0.00057726	112.2	90.9	High
IMPACT	1.552	0.00888483	431.9	93.4	High
MAGOHB	1.547	0.02824495	106.8	71.8	High
ITPA	1.538	1.6608E-05	166.1	88	High
UBE2I	1.521	0.00011548	99.9	89.3	High
FADD	1.507	0.01767791	156.1	87.2	High
LCN2	1.475	0.03656515	142.8	99.5	High
PLIN2	1.466	0.00026487	174.9	99.7	High
SPCS1	1.458	0.01759851	149.8	98.6	High
GOLT1B	1.456	0.02208771	291.2	200	High
TOR3A	1.455	0.00479504	225	99.8	High
RAB1A	1.446	6.3662E-05	127.2	97	High
ASAP2	1.441	0.00188561	139.5	96.8	Medium
NME1	1.44	0.01178699	135.8	91.5	High
MRPS33	1.411	0.02797597	121.7	86.2	High
NCLN	1.375	0.01291237	121.1	93.2	High
FTL1	1.367	0.00076046	126	99.8	High
CD14	1.362	0.00123337	140.7	99	High
APBB1IP	1.36	0.007835	118.5	99.4	High
PDXK	1.33	0.00336111	167.8	96.8	High
CD44	1.322	0.00536522	127.1	99.8	High
KRT10	1.309	0.01368055	125.9	99.9	High
RTRAF	1.301	0.01533075	214.8	96.2	High
RPLP2	1.299	0.0114497	113.1	96.8	High
MKLN1	1.294	0.03994319	135.7	97.8	High
RRBP1	1.29	0.03679254	512.5	98.6	High
MTX2	1.289	0.03616002	136	100	High
ANXA7	1.281	0.01450512	120.1	99.6	High
RHOC	1.27	0.02170953	122.6	98.8	High
PAM16	1.264	0.04296765	106.2	95.8	High
EIF4G2	0.733	0.04401257	72.9	99.8	High
WDR77	0.726	0.04208133	62.6	97.8	High
IRF5	0.714	0.04598378	59.9	94.3	High
ADH7	0.711	0.04108169	41.9	91.8	High
DDRGK1	0.709	0.04879336	70.3	99.2	High
BAG1	**0.704**	**0.02914574**	**70.1**	**98.8**	**High**

ABCF1	0.701	0.0244954	71.4	99.4	High
CORO1A	0.697	0.0196878	85.7	99.8	High
PLXNA1	0.691	0.04926876	80.5	99.6	High
TRMT6	0.673	0.03134088	61	85.8	High
FUBP3	0.672	0.03100634	66.3	100	High
PLOD3	0.662	0.032238	47.3	99.8	High
MFF	0.655	0.02115987	76.4	98.7	High
PGAM2	0.644	0.02505507	60.8	94.4	High
SRBD1	0.623	0.04982566	57.1	97.4	High
MOSPD2	0.603	0.04015537	59	98.3	High
ANKRD52	0.594	0.00359188	22.4	72	High
PGPEP1	0.592	0.01579369	75.6	99.2	High
TAF6L	0.586	0.00057726	56.1	95.8	High
NUP35	0.575	0.00104783	81.2	98.1	High
ZCCHC8	0.566	0.00575964	35.5	95.3	High
UBLCP1	0.565	0.04201333	74.1	93.5	High
FOXK1	0.559	0.02299553	49.2	99.8	High
DPH6	0.559	0.02691072	64.7	98.8	High
MAN2B1	0.546	0.00019801	39.9	99.7	High
L2HGDH	0.535	0.00131231	53.9	94.5	High
TMX4	0.534	0.00109429	112	90.7	High
NUP62	0.522	0.01023364	104.4	200	High
TNRC6A	0.515	0.000758	103	200	Low
BMP2K	0.515	0.01249039	48.7	94.6	High
EPM2AIP1	0.505	0.01359808	44.5	88.1	High
RNF31	0.505	0.0263966	101.1	200	High
FYCO1	0.49	0.01193106	108.2	200	High
CFL2	0.482	0.00315836	47.9	99.5	High
SCCPDH	0.479	0.00789065	46.2	96.5	High
RCOR1	0.478	0.00720649	97.9	200	High
POLR1B	0.476	0.01291237	95.3	200	High
ABI3	0.468	0.02451673	93.5	200	High
RPL39	0.467	0.00052986	46.7	100	High
WLS	0.458	8.0023E-05	57.2	97.1	High
FTO	0.458	0.03482247	44.4	97	High
IRF3	0.455	0.00614554	47	200	High
SLC11A2	0.452	0.0042217	18.8	41.7	High
UBE2F	0.452	0.0097351	45.4	61.1	High
ATP11C	0.451	0.0239151	90.2	200	High
MFN1	0.447	0.0001755	92.3	200	High
ARID3A	0.44	0.02942096	88	200	Medium
BOP1	0.426	0.00107554	138.1	93.6	High
CROT	0.406	0.02942096	81.2	200	High
AHSA2	0.401	1.4965E-05	80.1	200	High
UNC93B1	0.4	0.0001291	118.8	79.1	High
PDE4DIP	0.399	0.00767187	21.5	53.8	Low
CNOT11	0.398	0.00498304	39.3	98.7	High
KIAA1210	0.398	0.04505673	79.7	200	Low
DVL2	0.397	0.04251296	79.4	200	Low
TUT4	0.396	0.00214946	79.2	200	High
FNDC3A	0.392	0.00012622	19.1	82.7	High
STX16	0.391	0.03133431	39	99.6	High
DHRS13	0.39	0.01417139	78	200	Medium
STK32C	0.389	0.0186762	77.8	200	Low
CDR2L	0.387	0.02926357	38.7	100	Low
RLIM	0.383	0.00616311	76.7	200	High
HIGD1A	0.37	0.00175832	31.7	85.6	High
CCS	0.364	0.00324566	32.3	99.4	High
NUMA1	0.361	4.5635E-06	72.2	200	High
MED6	0.357	0.01341401	71.5	200	Low
CYB5B	0.343	3.7474E-08	32.8	95.6	High
HNRNPDL	0.339	0.00113307	29.4	86.7	High
N4BP1	0.339	0.00226294	20.3	59.8	High
PCDHGB7	0.338	0.02286708	33.5	99	Medium
POLR2K	0.337	0.00090881	128.5	200	High
ARF3	0.336	0.00019141	67.1	200	High
CREG1	0.333	5.677E-16	169.2	200	High
PCBD2	0.329	0.00385586	65.7	200	High
PIGB	0.327	0.00033674	65.3	200	High
NDUFAF1	0.314	0.00391507	62.7	200	High
RDH14	0.298	0.00026142	59.6	200	High

FLRT2	0.294	8.0067E-10	58.7	200	High
JPT1 HN1	0.286	0.00011977	57.2	200	High
AKAP10	0.281	5.1892E-06	56.2	200	High
SEPSECS	0.281	0.00296611	27.2	96.8	High
IRF2BP1	0.279	0.00065402	27	96.7	High
ATP6V0D2	0.268	6.7051E-05	20.3	75.9	High
SUPT20	0.262	9.3207E-06	52.5	200	High
UXT	0.26	5.677E-16	9	94.9	High
GBA2	0.258	0.00017201	51.5	200	Medium
UBE2Z	0.25	5.677E-16	24.8	99.5	High
PRKCI	0.245	2.9805E-06	24.4	99.6	High
SLC38A2	0.242	9.9362E-09	48.5	200	High
DYM	0.242	8.8521E-06	48.4	200	High
CASP9	0.239	8.24E-07	501.4	200	High
TAS2R106	0.231	0.00023062	8.5	45.7	High
TRIM56	0.226	6.4684E-13	45.2	200	High
METTL3	0.219	1.109E-07	19.3	88.2	High
MAP2K2	0.215	5.677E-16	126.9	200	High
FAM20B	0.21	5.677E-16	7.5	48.5	High
ORMDL1	0.207	1.8884E-08	41.3	200	High
PANX1	0.207	1.9715E-07	14.5	70.2	High
KRAS	0.189	5.677E-16	37.8	200	High
KDELR2	0.16	2.1261E-11	32	200	High
KRT79	0.157	5.677E-16	31.4	200	High
EBP	0.157	5.677E-16	15.7	99.9	High
TMEM132D	0.138	6.3876E-13	27.6	200	Low
PRCC	0.121	3.5961E-11	24.2	200	High
SYNE2	0.117	3.6271E-14	23.4	200	High
TRIP10	0.104	5.677E-16	24.1	97.6	High
CACNA1I	0.102	5.062E-11	20.4	200	Medium
HHIPL2	0.096	5.677E-16	19.1	200	Medium
UBR1	0.086	5.677E-16	32.7	200	High
OLFR607	0.057	5.677E-16	1.8	31.8	Low
TRMT44	0.033	5.677E-16	3.3	99.5	High

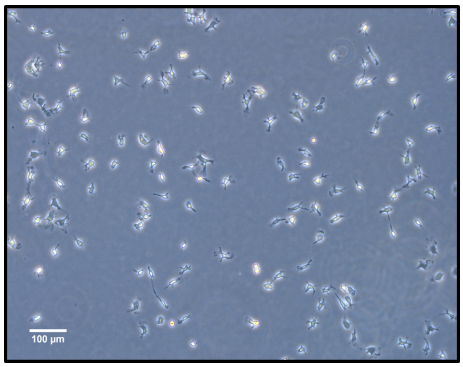
Supplementary Figure 1



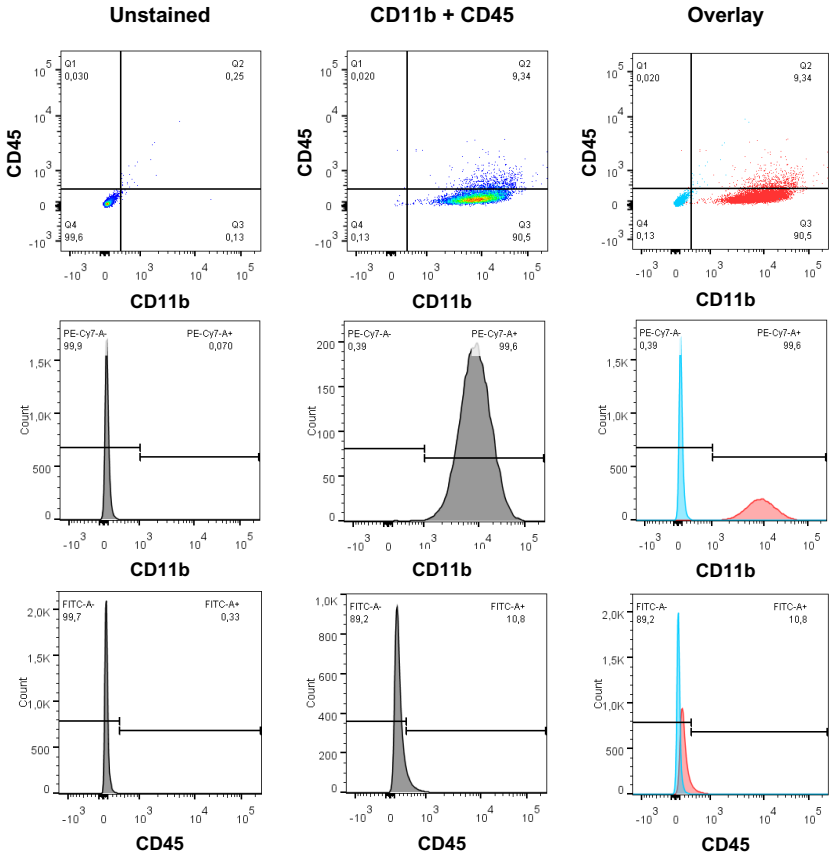
Supplementary Figure 1. Rat microglia isolation and in vitro stimulation procedures. (i) Newborn rat pups (P1-P2) were decapitated and brains separated from the skull; (ii) Cerebellum and meninges were carefully removed for efficient brain dissection. Dissected tissue was treated with DNase and trypsin before being dissociated and plated; (iii) Mixed glial cells (astrocytes, oligodendrocytes and microglia) were cultured with 10% FBS supplemented media in T75 poly D-lysine coated flasks for 21 days; (iv) Microglia were obtained at day 14 and 21 of culture by shaking the flasks at 37°C for 2h at 150 rpm; (v) Isolated microglia were re-seeded in 6-well plates, allowed to adhere for 48h and then stimulated for 6h with TNF-α (20 ng/mL) or LPS (100 ng/mL); (vi) Activated microglia were finally harvested for RNA or protein extraction.

Supplementary Figure 2

A.

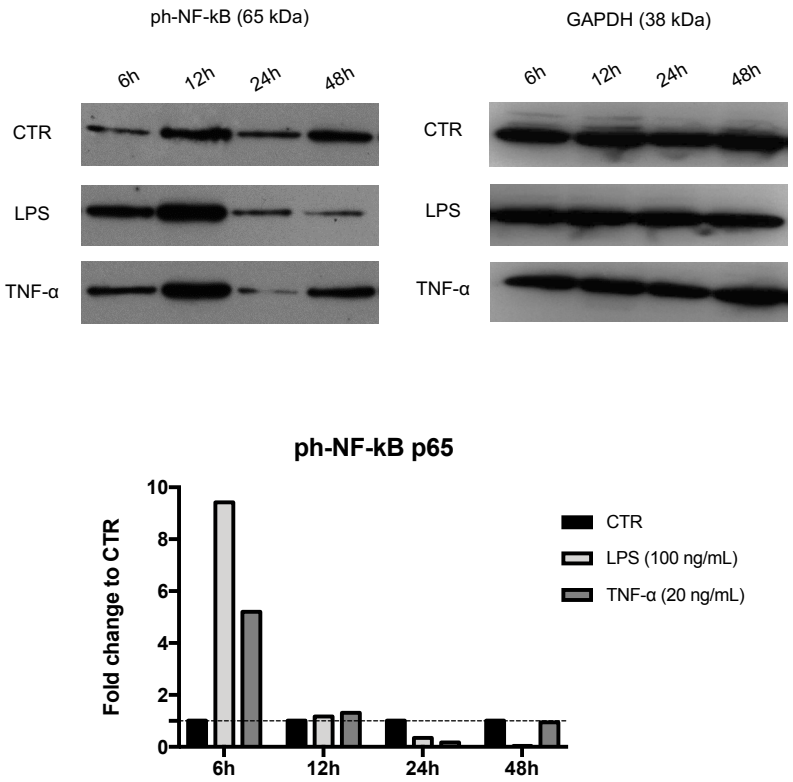


B.



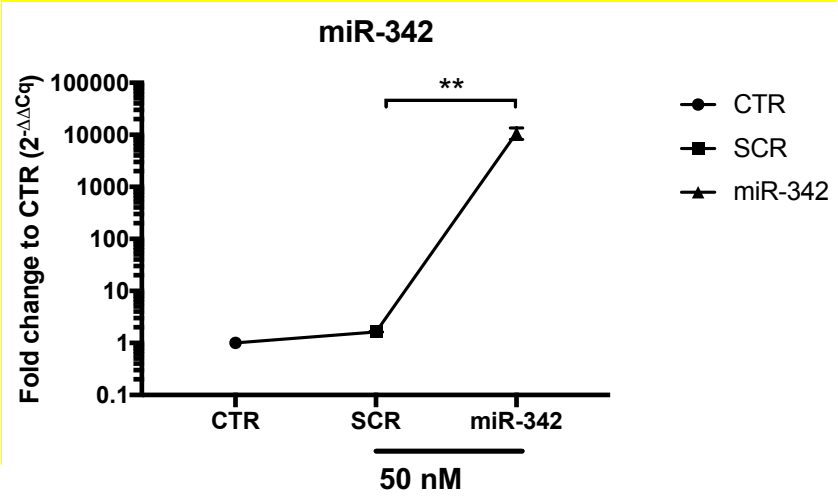
Supplementary Figure 2. Microglia characterization by flow cytometry. (A) Microscopy image of rat microglia, 48h after being isolated from mixed glial culture and re-seeded (scale bar, 100 µm). Dot plots and histograms represent microglia surface expression evaluation by flow cytometry. After every isolation, more than 95% of the isolated cells were CD11bhigh/CD45low, corresponding to microglia surface expression signature

Supplementary Figure 3



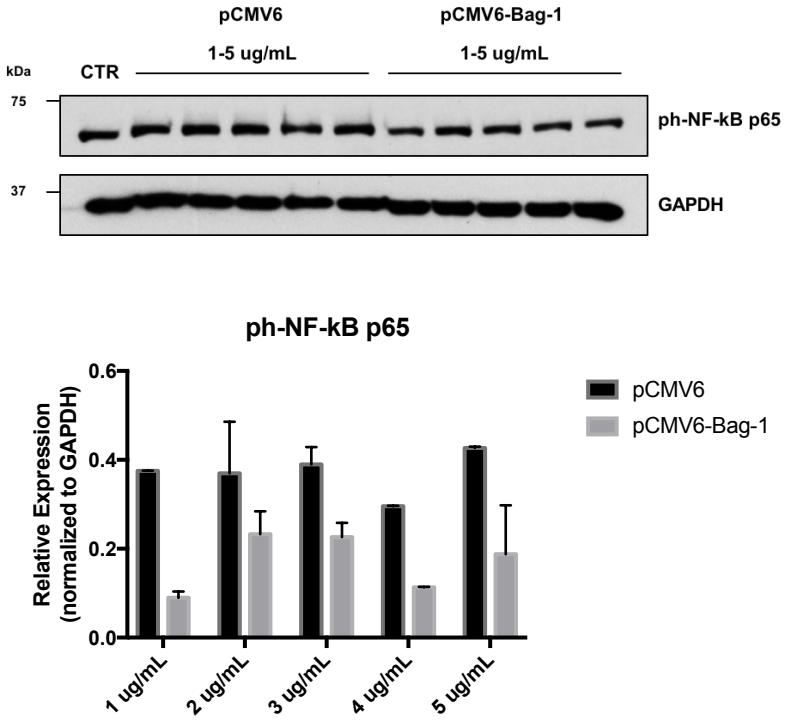
Supplementary Figure 3. Rat microglia ph-NF-κB p65 peak expression after LPS and TNF-α stimulation occurs at 6h. Preliminary experiment performed to optimize the duration of stimulation needed to achieve ph-NF-κB p65 maximum expression, comparing to non-stimulated microglia. Rat microglia were obtained from mixed glial cultures. Isolated microglia were re-seeded in 6-well plates, allowed to adhere for 48h and then stimulated for 6h, 12h, 24h and 48h with LPS (100 ng/mL) or TNF-α (20 ng/mL).

Supplementary Figure 4



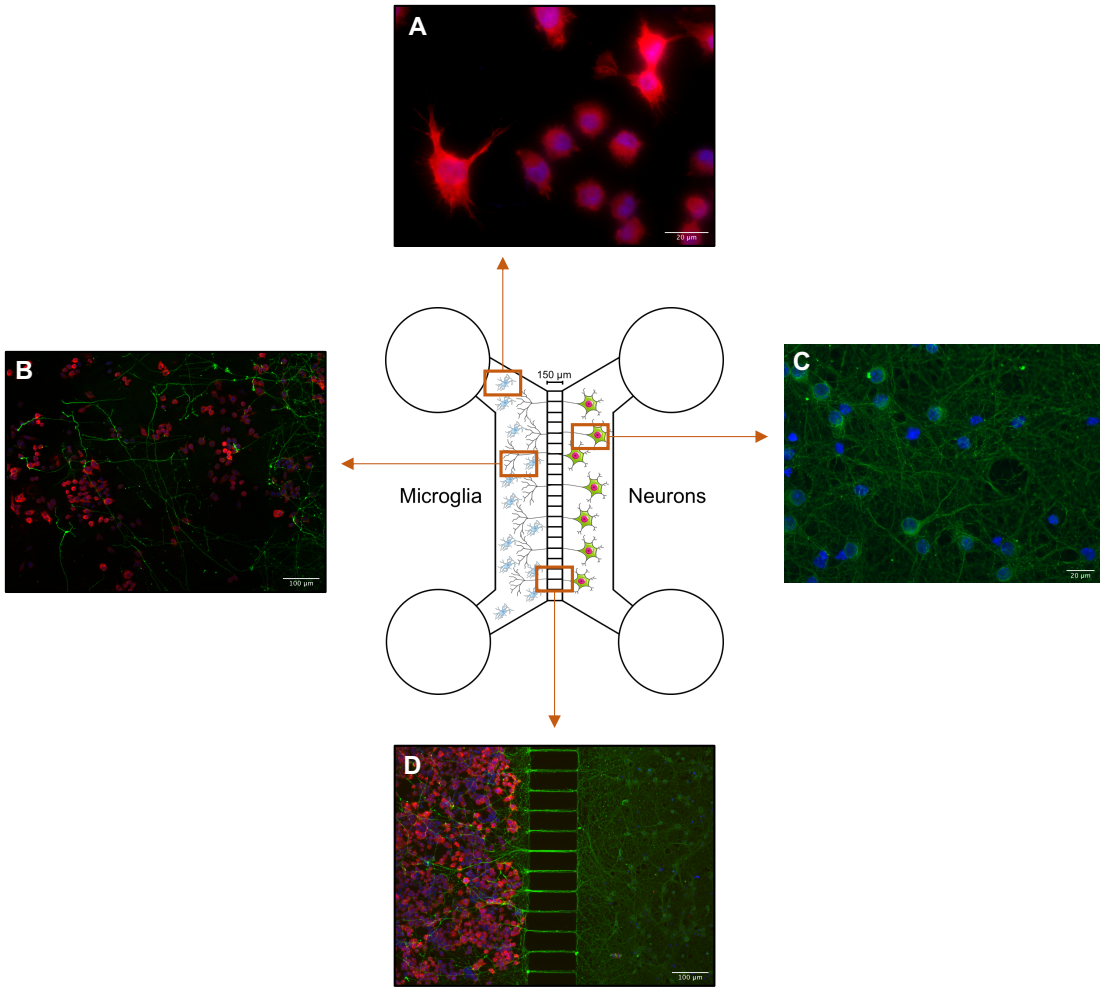
Supplementary Figure 4. Transfection efficiency of miRVana mmu-miR-342-3p mimics in N9 microglia, using Lipofectamine 2000. Transfection efficiency was evaluated by RT-qPCR and compared with the controls (n=2). Statistical significance: **p<0.001.

Supplementary Figure 5



Supplementary Figure 5. ph-NF-κB p65 expression after pCMV6-Bag-1 transfection. Preliminary titration (1-5 ug/mL) was performed in order to select pCMV6-Bag-1 concentration for the overexpression experiments (n=2). 1 ug/mL of pCMV6-Bag-1 was the concentration that induced the highest reduction on ph-NF-κB expression levels comparing with the pCMV6 empty control, and therefore it was used for the remaining experiments.

Supplementary Figure 6



Supplementary Figure 6. Hippocampal Neurons-N9 microglia co-culture in the Axon Investigation System. (A) N9 microglia cells stained with anti-IBA1 (anti-Alexa 594, red). (B) Left side of the device containing N9-microglia in direct contact with axons. (C) Right side of the device containing neuron cell bodies stained with anti-β3-tubulin (anti-Alexa 488, green). (D) Axon Investigation System microgrooves (150 µm length) through which neurons project their axons and communicate with microglia. Cell nuclei were stained with Hoechst (Blue).

Supplementary Figure 7

>mmu-miR-342-3p MIMAT0000590

UCUCACACAGAAAUCGCACCCGU

>rno-miR-342-3p MIMAT0000589

UCUCACACAGAAAUCGCACCCGU

Supplementary Figure 7. miR-342-3p sequences in mouse and rat. miR-342-3p sequences for mouse and rat were obtained in miRbase (www.mirbase.org). miR-342-3p sequence has 100% homology between both species.

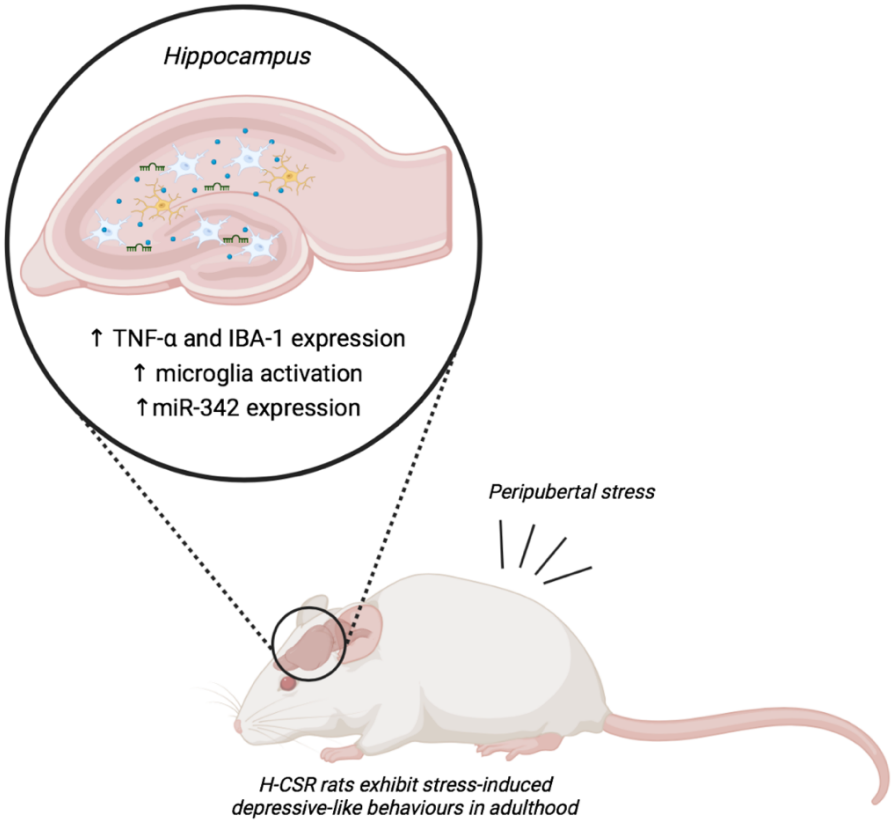
2. Stress-induced depressive-like behaviour in male rats is associated with microglial activation and inflammation dysregulation in the hippocampus in adulthood

The content of this chapter was compiled in a manuscript that is currently under review.

Article III

João P. Brás, Isabelle G.de Suduiraut, Olivia Zanoletti, Silvia Monari, Mandy Meijer, Jocelyn Grosse, Mário A. Barbosa, Susana G. Santos, Carmen Sandi, Maria Inês Almeida. **Stress-induced depressive-like behaviour in male rats is associated with microglial activation and inflammation dysregulation in the hippocampus in adulthood.** *Under review in an international peer reviewed journal*

Graphical abstract



Graphical abstract. Stress-induced depressive-like behaviour in male rats is associated with microglial activation and inflammation dysregulation in the hippocampus in adulthood. In this study, we show that peripubertal stress, combined with high-CORT stress responsiveness (H-CSR), potentiate the development of depressive-like behaviours and neuro-immunological alterations in adulthood, particularly increased microglial activation, TNF-α and miR-342 expression levels in the hippocampus.

Abstract

Neuroinflammation is increasingly recognized as playing a critical role in depression. Early-life stress exposure and constitutive differences in glucocorticoid responsiveness to stressors are two key risk factors for depression, but their impacts on the inflammatory status of the brain is still uncertain. Moreover, there is a need to identify specific molecules involved in these processes with the potential to be used as alternative therapeutic targets in inflammation-related depression. Here, we studied how peripubertal stress (PPS) combined with differential corticosterone (CORT)-stress responsiveness (CSR) influences depressive-like behaviours and brain inflammatory markers in male rats in adulthood, and how these alterations relate to microglia activation and miR-342 expression. High-CORT stress-responsive (H-CSR) male rats that underwent PPS exhibited increased anhedonia and passive coping responses in adulthood. Also, animals exposed to PPS showed increased hippocampal TNF-α expression, which positively correlated with passive coping responses. In addition, PPS caused late effects on hippocampal microglia, particularly in H-CSR rats, with increased hippocampal IBA-1 expression and morphological alterations compatible with a higher degree of activation. H-CSR animals that underwent PPS, also showed upregulation of hippocampal miR-342, a mediator of TNF-α-driven microglial activation, and its expression was positively correlated with TNF-α expression, microglial activation and passive coping responses. Our findings indicate that individuals with constitutive H-CSR are particularly sensitive to developing protracted depression-like behaviours following PPS exposure. In addition, they show neuro-immunological alterations in adulthood, such as increased hippocampal TNF-α expression, microglial activation and miR-342 expression. Our work highlights miR-342 as a potential therapeutic target in inflammation-related depression.

Keywords: Peripubertal stress, corticosterone, neuroinflammation, cytokines, microRNAs

2.1. Introduction

The immune system is emerging as a key player in depression symptomatology and treatment resistance (1-3). A segment of depression patients present with hyperactivation of the immune system (4), including increased levels of systemic and brain proinflammatory mediators, such as TNF-α, IL-6 and IL-1β (5-9); trafficking of immune cells to the brain; and activation of microglia (10, 11). Specifically, increased levels of TNF-α have been shown to impact neurocircuits and cause neuronal damage directly, and indirectly, by overstimulating astrocytes and microglia (12, 13). Activated microglia is characterized by the of release cytotoxic molecules, including TNF-α, produced by a positive feedback of autocrine activation (14, 15). The mechanisms underlying the dysregulation of the immune system in depression-like behaviours have been studied in rodents, particularly using peripheral administration of lipopolysaccharide (LPS) (16). However, from a translational perspective, it is not clear whether and how well the behavioural effects of LPS injection and the magnitude of the resulting inflammatory response “translate” to the clinic and compare to human depression (17). Therefore, the use of alternative animal models focusing on risk factors that naturally increase the susceptibility to depression development is warranted, rather than models established by exacerbated induction of systemic and local inflammation.

Risk factors for depression include early-life stress exposure and constitutive differences in glucocorticoid responsiveness to stressors (18, 19). Over the last decade, the impacts of these factors in the context of inflammation-related depression have received increasing attention (20, 21). The peripubertal period, comprising childhood and adolescence, is a critical time window in brain development that is sensitive to the deleterious effects of adverse experiences. An enhanced risk of developing depression in adulthood following stress exposure in early life has been reported in several studies (22, 23). In our laboratory, we have shown that peripubertal stress (PPS) in rats leads to enhanced anxiety-related behaviours and increased passive stress coping responses, a key symptom in depression (18, 24). Recent findings have shown that repeated social defeat stress in adulthood leads to increased neuroinflammation and microglial activation in rats (25). However, little is known about the long-term effect of early-life stressful events on the brain inflammatory status in adulthood.

Glucocorticoids coordinate responses that enable an individual to cope with stressful challenges, mediating adaptation following a stressor’s cessation (26). However, there is substantial individual variability in the magnitude of glucocorticoid responsiveness to stressors, a trait highly related to differences in coping styles (19). Recently, the view that glucocorticoids are universally anti-inflammatory has been questioned (27, 28). Persistent activation of the hypothalamus-pituitary-adrenal (HPA) axis was shown to lead to downregulation of glucocorticoid receptors involved in the negative feedback controlling the

HPA axis (29). This downregulation has been associated with a failure to suppress inflammatory responses (30). We previously established a selective breeding protocol that generates lines of rats enriched for different levels of corticosterone (CORT)-stress responsiveness (CSR) (19). However, it is not known whether early-life stress differentially affects individuals who show differences in glucocorticoid stress responsiveness, in terms of protracted inflammatory marker expression and microglial activation in the brain, nor are potential correlations with indexes of depression clear. Uncovering the regulatory mechanisms governing these processes can provide new targets for the development of alternative therapeutic strategies.

In this context, microRNAs (miRNAs), which are small noncoding RNAs (approximately 20 nucleotides long) that control the expression of multiple protein targets (31), have been implicated in several mechanisms of neural plasticity, neurogenesis, stress, antidepressant treatment response and neuroinflammation (32-34), providing strong evidence that miRNAs not only can play critical roles in depression pathogenesis but are also potential therapeutic tools. Recently, we reported that miR-342 is a crucial player in TNF-α-mediated microglial activation (35). miR-342 is upregulated in TNF-α-activated microglia, and its overexpression activates, *per se*, the NF-κB pathway, leading to increased secretion of TNF-α and IL-1β, which drastically affects neuron viability (35). However, it remains to be determined how depression-like behaviours correlate with the expression levels of miR-342, particularly in the brain.

The aim of the study was to investigate how PPS and different CORT adaptations to stress impact depressive-like behaviours, inflammatory markers across different brain regions, microglial activation and miR-342 expression in adulthood. We showed that animals with high-CSR that underwent PPS exhibited increased hippocampal microglial activation and depressive-like behaviours in adulthood, which were associated with dysregulated expression of inflammatory markers, including miR-342, in the hippocampus.

2.2. Materials and methods

2.2.1. Animals

Subjects were the offspring of Wistar Han rats (Charles River Laboratories, France) bred in the animal facility of the EPFL/SV CGP *(Centre de PhénoGenómique)*. Male rats were maintained under standard housing conditions on a 12-h light-dark cycle (lights on at 7:00 am). Food and water were available ad libitum. Animal care procedures were conducted in accordance with the Swiss Federal Guidelines for Animal Experimentation and were approved by the Cantonal Veterinary Office Committee for Animal Experimentation (Vaud, Switzerland).

2.2.2. Experimental design

Selective breeding of rats according to their individual differences in CSR was performed as previously described by Walker *et al.* (19). This resulted in litters (generation F14) of N-CSR (inter-line) and H-CSR (high-line). At weaning i.e., postnatal day 21 (P 21), male rats from both groups were randomly assigned to control (CTR) and PPS conditions. Animals were distributed into home cages in groups of three non-siblings. Between P 28 and P 42, animals were maintained in control conditions (CTR group) or subjected to the PPS protocol (PPS group) (Figure 1A). In adulthood (P 90), CTR and PPS rats underwent five sequential behavioural tests for anxiety (elevated plus-maze and open field/novel object), sociability (social preference), and depressive-like behaviour (saccharin preference and forced-swim test) with at least 3 days of interval between each test (Figure 1A). All behaviour tests were performed in the morning. In total, 42 animals were used, namely 18 N-CSR (9 CTR and 9 PPS) and 24 H-CSR (12 CTR and 12 PPS). The experimenter was blind during both testing and analysis.

2.2.3. Peripubertal stress

The PPS protocol was based on exposure to fear-induction procedures, according to Marquez *et al.* (24). The stressors were applied during the peripubertal period (a total of 7 days across P 28 to P 42) during the light phase and followed a variable schedule. Animals in the same cage were always assigned to the same experimental group (either CTR or PPS). Briefly, following exposure to an open-field for 5 min on P 28, the stress protocol consisted of presenting two different fear-inducing stressors, each one lasting for 25 min. Animals were either exposed to the synthetic fox odour 2,4,5-Trimethylthiazole (TMT, Sigma-Aldrich, St. Louis, MO, USA) administered in a plastic box (38 × 27.5 × 31 cm) or to an elevated platform (EP) (12 × 12 cm) under bright light, presented alone or in combination. To assess the effect of stress exposure on the HPA axis activity, blood samples from PPS animals were collected by tail-nick (100–150 µl) at P 28, P 30, and P 42. The sampling was done immediately after stress offset and then 30 min after the first blood sampling. During this interval, rats were placed in a novel cage and were prevented of direct physical contact with their cage mates. After the second blood sampling, animals were placed back in their home cage. CTR rats underwent brief handling on stress days, no blood samples were taken.

2.2.4. Evaluation of corticosterone levels

Blood samples were collected into ice-cold lithium heparin-coated capillary tubes (Sarstedt, Nümbrecht, Germany) and chilled on ice until centrifugation for plasma collection (10,000 rpm at 4°C for 4 min). Plasma was collected into new tubes and stored at −20ºC until subsequent

analysis. CORT was measured in the plasma samples using an enzymatic immunoassay kit, performed according to manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA). Levels were calculated using a standard curve method.

2.2.5. Behavioural testing

Elevated plus maze. In adulthood, anxiety-like behaviour was evaluated using the elevated plus-maze (EPM) test. The apparatus consisted of two opposing open arms (50-10 cm) perpendicular to two enclosed arms (50-10-50 cm) extending from a central platform (10-10 cm) and elevated 65 cm above the floor. Light levels were maintained at 14–16 lx on the open arms and 5–7 lx on the closed arms. At the start of the test, rats were placed in the centre of the platform facing a closed arm and allowed to explore the maze for 5 min. Between animals, the apparatus was cleaned with a 5% ethanol solution and dried. Behaviour was monitored using a ceiling-mounted video camera and analysed with the computerized tracking system EthoVision 11 (Noldus IT). The time spent in the open and closed arms and the distance moved were recorded.

Open field/Novel object. Anxiety-related behaviour was tested in the open-field (OF) and novel object (NO) test, as previously described (36). The open field consisted of a black pool with a diameter of 1 m and a depth of 40 cm. The floor of the pool was divided into three zones: the outer zone with a diameter of 1 m, the inner zone with a diameter of 75 cm, and the centre zone with a diameter of 25 cm. The light was adjusted to a level of 7 lx in the centre of the pool. Animals were placed close to the wall of the pool, and open-field activity was tested for a 10 min period. Subsequently, a novel object was introduced into the centre of the pool, and rat behaviour was observed during the following 5 min. The activity and behaviour during the whole session were recorded with a video camera, and the time spent in each of the zones (wall, intermediate zone, and centre) was automatically registered and analysed with the computerized tracking system EthoVision 11.

Social preference test. The social preference test (SocPT) was performed as previously described (24). Briefly, the test was performed in a rectangular, three-chambered box that included a central compartment and two side compartments. After 5 min of habituation to the central chamber, retractable doors were removed, and the rat was allowed to freely explore the whole apparatus for 10 min. The side compartments were equipped with a central, floor-fixed, transparent, perforated cylinder that contained either an unfamiliar juvenile male rat (25 ± 2 days old) or an object. The apparatus was cleaned with a 5% ethanol solution and dried between trials. The percentage of time spent exploring either the juvenile or the object was scored using Observer X11 software (Noldus IT, Wageningen, The Netherlands).

Saccharin preference test. Behavioural anhedonia was evaluated using the saccharin preference test (SacPT). Experimental and control groups were tested at the same time, and all groups received two days of habituation to the new bottle configuration (two bottles filled with tap water placed in the home cage for 24 h) prior to the test. A two-bottle choice procedure was used to determine baseline saccharin intake. During the test, single-housed rats were presented with two bottles (water and a 0.03% saccharin solution) in their home cage for a period of 72 h. The difference in bottle weight was determined (intake) every 24 h, and the bottle locations were counterbalanced. Saccharin preference was calculated as saccharin intake per total fluid intake (water + saccharin).

Forced-swim test. Rats were submitted to a forced-swim test (FST) to evaluate depression-like behaviour, following previously described conditions (24). Animals were placed in a plastic beaker (25-cm diameter x 46 cm) containing 30 cm of water (23-25°C) for 15 min. A second session was performed 24 h later for 5 min. Both sessions were recorded using video cameras mounted in front of the beakers, and the time spent immobile, swimming or diving was quantified manually in a blinded manner using Observer X11 software.

2.2.6. RNA extraction

After rats were sacrificed by decapitation, the brain was rapidly removed, and the hemispheres were divided. Left hemispheres were freshly dissected for isolation of the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), amygdala (Amg) and hippocampus (HPC). Each brain region was placed in an RNase-free cryotube, fresh frozen in liquid nitrogen, and stored at -80°C prior to RNA extraction. Total RNA was extracted from brain sections using TRIzol® reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. RNA concentration was evaluated using a NanoQuant Plate™ in a Spark® multimode microplate reader (Tecan, Männedorf, Switzerland). RNA integrity was evaluated by agarose gel electrophoresis.

2.2.7. Gene expression analysis

For gene expression analysis, RNA was treated with the TURBO DNA-free™ Kit (Invitrogen), and cDNA was synthesized using qScript® SuperMix (Quanta Biosciences, Beverly, MA, USA) according to the supplier's recommendations. Real-time quantitative polymerase chain reaction (qPCR) was carried out on a 7900HT Fast Real-Time PCR System with the 384-Well Block Module (Applied Biosystems, Waltham, MA, USA) using cDNA, primers (Microsynth, Balgach, Switzerland) and SYBR™ Green PCR Master Mix (Applied Biosystems™). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (β-actin) were used as internal controls. The expression of IBA-1 (a microglial marker) and the

inflammation-related cytokines IL-1β, IL-6 and TNF-α was evaluated. The oligonucleotides used for qPCR experiments are shown in Table S1.

2.2.8. miRNA expression analysis

miR-342-3p expression was evaluated using TaqMan™ microRNA assays (Applied Biosystems™). Briefly, cDNA was synthesized using 30 ng of RNA as the template, a gene-specific stem-loop reverse transcription primer, and a TaqMan™ microRNA reverse transcription kit (Applied Biosystems™). qPCR was carried out on a CFX Real-Time PCR System with the 384-Well Block Module (Bio-Rad, Hercules, CA, USA) using cDNA, a TaqMan™ probe and SsoAdvanced™ Universal Probes Supermix (Bio-Rad). The small nuclear RNA U6 was used as a reference gene. Relative expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines (37).

2.2.9. Immunofluorescence

Right hemispheres were postfixed for 24 h in 4% PFA at 4°C, incubated for 48 h in a 30% sucrose solution at 4°C, flash frozen in isopentane and stored at -80°C before being further processed. Frozen brains were cut into 40-µm coronal sections with a cryostat (Leica, Wetzlar, Germany). Brain sections were blocked in PBS with 3% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.3% Triton X-100 (Sigma-Aldrich) for 1 h at room temperature (RT) and incubated overnight at 4°C with goat anti-Iba-1 (ab5076, Abcam, Cambridge, UK) and rabbit anti-NeuN (ABN78, Merck Millipore, Burlington, MA, USA) primary antibodies (1:500) in PBS plus 1% donkey serum and 0.1% Triton X-100. Fluorophore-labelled anti-goat (1:500, AlexaFluor-568) and anti-rabbit (1:500, AlexaFluor-488) IgGs were used as secondary antibodies, and DAPI (1:10000 in PBS) was used to stain the nuclei (10 min at RT). Finally, sections were mounted on SuperFrost Plus™ Adhesion slides (Thermo Scientific, Waltham, MA, USA) with Fluoromount-G® mounting medium (Southern Biotech, Birmingham, AL, USA).

2.2.10. Microglial morphological analysis

Images of three different subregions (CA1, CA3 and dentate gyrus (DG)) in the dorsal hippocampus were acquired using an upright confocal laser scanning microscope (Leica SP8) with a 40x glycerine immersion objective. The pixel size was set to 0.230 microns with a z-step of 0.5 microns. The acquired images were deconvolved using the SVI Huygens Professional (Scientific Volume Imaging B.V., Hilversum, The Netherlands) software program called via Huygens Remote Manager v3.7, which uses the “Classic Maximum Likelihood Estimation” algorithm (SNR = 5, quality change = 0.001, max. iterations = 50). Analysis of the deconvolved images was performed with Fiji (38) and Imaris (BitPlane, South Windsor, CT,

USA) using the EasyXT-Fiji (https://github.com/BIOP/EasyXT-FIJI) plugin via a custom groovy script. Briefly, Imaris surfaces were created to segment the arborization of cells (smoothing=0.230, threshold=7) and for their soma (smoothing=1, threshold=10). Next, cells were created by linking each soma to its corresponding arborization based on the nearest centers of mass. Next, for each cell, the mask of the arborization was sent to Fiji, 1) skeletonized and analysed (average and maximum branch lengths, number of branches), and 2) down sampled to calculate its 3D Convex Hull (39), which was then upscaled back to the original size. Finally, the masks of the Skeleton and 3D Convex Hull were sent back to the Imaris Scene, and a results file was created with volumes and calculated ratios. This approach was chosen to avoid possible biases induced by a z-axis maximum intensity projection used to render 3D data in 2D.

2.2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Gaussian distribution was tested by the D'Agostino & Pearson and Shapiro-Wilk normality tests. For normally distributed datasets, an unpaired *t*-test was used to identify significant differences between only two groups. When analysing the difference between the means of more than two groups, 2-way ANOVA was performed to estimate how the mean of the quantitative variables changed according to the levels of the independent variables (CSR and stress exposure). To test which levels were actually different between the CTR and PPS groups, Sidak's post hoc multiple comparisons test was performed. Pearson correlation analyses were performed considering all animals, and the resulting r and *p*-values are represented in each plot. All bars and error bars represent the mean ± SEM. The sample size and the statistical tests used are indicated in each figure legend. $p<0.05$ was considered statistically significant.

2.3. Results

2.3.1. Exposure to peripubertal stress gives rise to dissociable alterations in social and depressive-like behaviours in adulthood

After weaning, animals were exposed to a PPS protocol based on fear-induction procedures between P 28 and P 42 (Figure 1A). During the PPS protocol, CORT levels were measured in plasma samples collected on P 28, P 30, and P 42. Analysis of the CORT concentration revealed a significant interaction between selection CSR groups and sampling timepoints (2-way RM ANOVA: $F_{(2,38)} = 5.937$, $p = 0.0057$, Figure 1B). In accordance with our previous data (19, 40), after the first stressor exposure (P 28), the N-CSR and H-CSR groups did not differ in their CORT response (2-way RM ANOVA post hoc test: P $28_{(N\text{-}CSR\ vs\ H\text{-}CSR)}$: t = 1.389, $p = 0.428$) but showed significant differences on the third (P $30_{(N\text{-}CSR\ vs\ H\text{-}CSR)}$: t = 3.88, $p < 0.001$) and last days of the protocol (P $42_{(N\text{-}CSR\ vs\ H\text{-}CSR)}$: t = 4.84, $p < 0.001$), with H-CSR rats showing increased CORT levels compared with N-CSR rats (Figure 1B).

Anxiety-like behaviours

To address the impact of different CSRs and PPS on anxiety-like behaviours in adulthood, EPM and OF/NO tests were performed (Figure 1C and D). In the EPM test, no significant interactions were found between different CSRs and PPS exposure in the time spent in the CA ($F_{(1,38)} = 0.864$, n.s.) or in the OA ($F_{(1,38)} = 0.601$, n.s.). Neither different CSRs nor PPS exposure influenced the time spent in the CA/OA (Figure 1C). Additionally, the number of entries in zone and total distance moved in the EPM test were not affected by either different CSRs or PPS exposure (Figure S1A). Generally, animals spent more time in the protected closed arms than in the open arms of the maze, independent of their CSR or stress exposure ($F_{(3,76)} = 13.69$, $p < 0.001$; Figure 1C).

In the OF test, a significant interaction was observed between different CSRs and PPS exposure in the time spent in the wall ($F_{(1,38)} = 5.465$, $p = 0.025$) and intermediate zones ($F_{(1,38)} = 9.003$, $p = 0.0248$; Figure 1D). H-CSR rats submitted to the PPS protocol spent significantly more time in the wall zone (H-CSR $_{(CTR\ vs\ PPS)}$: wall: t = 2.351, $p = 0.047$) and less time in the intermediate zone than those in the H-CSR non-PPS control group (H-CSR $_{(CTR\ vs\ PPS)}$: inter: t = 3.077, $p = 0.007$; Figure 1D). Conversely, the time spent across the different zones by N-CSR rats was not affected by PPS exposure (Figure 1D). Of note, the total distance moved during the test was not affected by either different CSRs or PPS exposure (Figure S1B). After 10 min of open-field tracking, an NO was introduced into the centre of the pool, and the behaviour was recorded for an extra 5 min. The introduction of the NO induced high variability in the behavioural responses across all experimental groups, and no differences were found (Figure S1C).

Social behaviours

In the SocPT, the time spent exploring the juvenile was significantly impacted by both different CSRs ($F_{(1,38)} = 5.350$, $p = 0.026$) and PPS exposure ($F_{(1,38)} = 13.850$, $p < 0.001$; Figure 1E), although the interaction between both factors was not statistically significant ($F_{(1,38)} = 0.052$, n.s.). In fact, rats from both the N-CSR and H-CSR groups submitted to PPS interacted with the juvenile for significantly less time than the respective controls (N-CSR $_{(CTR\ vs\ PPS)}$: $t = 2.612$, $p = 0.025$; H-CSR $_{(CTR\ vs\ PPS)}$: $t = 2.669$, $p = 0.022$; Figure 1E). In addition to the influence on the time spent exploring the juvenile, PPS rats showed a reduction in total exploration (juvenile + object) ($F_{(1,38)} = 13.22$, $p < 0.001$; Figure S1D). These results show that PPS significantly decreases animals' motivation to engage in social exploration and interaction, independently of their CSR.

Depressive-like behaviours

Depressive-like behaviours were evaluated by SacPT and FST. Anhedonia evaluation in rodents takes advantage of their innate preference for sweets (41). The results showed that PPS exposure significantly impacted saccharin preference on the second ($F_{(1,38)} = 5.354$, $p = 0.026$) and third days of the test ($F_{(1,38)} = 4.832$, $p = 0.034$). Post hoc analysis revealed that H-CSR rats subjected to PPS had a significantly lower preference for saccharin than the respective control non-PPS rats (H-CSR $_{(CTR\ vs\ PPS)}$: day 2: $t = 2.603$, $p = 0.025$; day 3: $t = 3.085$, $p = 0.016$; Figure 1F). On the other hand, no significant differences were found in N-CSR rats when comparing the PPS and CTR groups (Figure 1E). Importantly, total consumption (water + saccharin) did not change among the groups (Figure 1E).

The FST is widely used to assess animals' coping behaviours. We exposed rats to a two-day forced-swimming procedure and quantified the time spent immobile. During the first episode of the FST, neither CSR ($F_{(1,38)} = 0.6479$, n.s.) nor PPS ($F_{(1,38)} = 0.0397$, n.s.) influenced animals' behavioural response in terms of time spent floating (Figure S1F). However, during the second exposure, the time spent floating was significantly increased by PPS exposure ($F_{(1,38)} = 7.696$, $p = 0.008$). Post hoc analysis revealed that H-CSR rats but not N-CSR rats (N-CSR $_{(CTR\ vs\ PPS)}$: $t = 1.449$, n.s.) that underwent PPS spent significantly more time floating than H-CSR non-PPS control rats (H-CSR $_{(CTR\ vs\ PPS)}$: $t = 2.564$, $p = 0.028$; Figure 1G). Therefore, H-CSR rats are more susceptible to PPS, exhibiting depressive-like behaviours in adulthood.

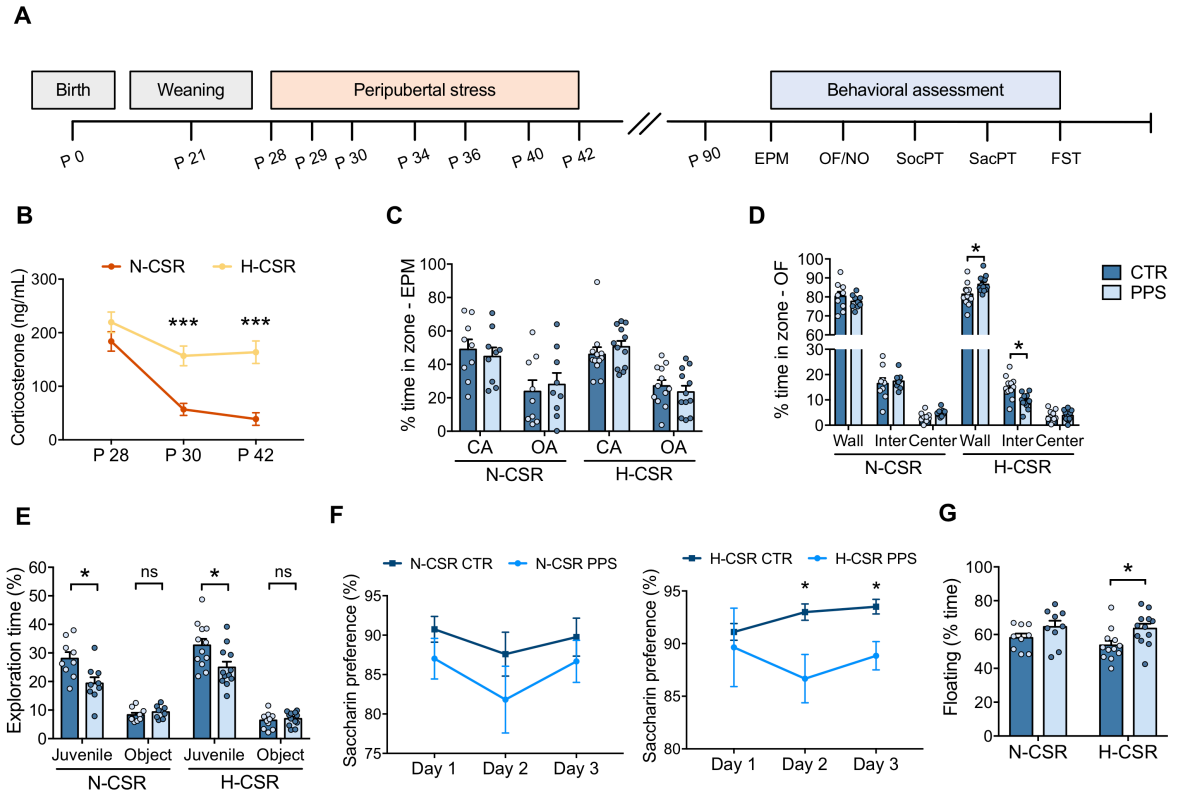


Figure 1. Effects of peripubertal stress on anxiety-like, social and depression-like behaviours in rats with differential CORT stress responsiveness in adulthood. (A) Experimental procedures and timeline. (B) Plasma CORT levels of normative- (N-CSR) and high-CORT stress responsive (H-CSR) rats after stress exposure at post-natal days (P) 28, P 30 and P 42. (C-D) In adulthood, animals were tested for anxiety-like behaviours using the elevated plus-maze (EPM) and open field/novel object (OF/NO) tests. (C) In the EPM, rats were placed in the centre of the platform facing a closed arm and allowed to explore the maze for 5 min. (D) In the OF test, animals were placed close to the wall of the pool, and their activity was monitored for 10 min. In both tests, the % of time spent in each zone was calculated using EthoVision 11. (E) In the social preference test (SocPT), rats were allowed to freely explore for 10 min a three-chambered box containing perforated cylinders with either an unfamiliar juvenile male rat or an object in the side compartments. The % of time spent exploring either the juvenile or the object was scored using Observer X11. (F-G) Rats were also tested for depressive-like behaviours using the saccharin reference test (SacPT) and forced-swim test (FST). (F) In the SacPT, single-housed rats were presented with two bottles (water and a 0.03% saccharin solution) in their home cage for 72 h. Every 24 h, the difference in bottle weight was determined (intake), and bottle location was counterbalanced. Saccharin preference (%, saccharin intake/total consumption) was calculated. (G) In the FST, two trials were performed, recorded and quantified manually in a blinded manner using Observer X11. The first trial lasted 15 min, and the second (24 h later) lasted 5 min. The graph represents the % of time spent floating/immobile in the second trial. N: N-CSR = 18 (9 CTR and 9 PPS); H-CSR = 24 (12 CTR and 12 PPS). Results are expressed as the mean ± SEM. Two-way ANOVA followed by Sidak's post hoc multiple comparisons test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s.: nonsignificant. CA - closed arms, OA - open arms.

2.3.2. Hippocampal TNF-α expression is enhanced by peripubertal stress and positively correlates with depressive-like behaviours in adulthood

Next, we evaluated the impact of PPS and different CSRs on proinflammatory markers across different brain regions known to be involved in the pathophysiology of depression. The expression levels of IL-1β, IL-6, TNF-α and IBA-1 were evaluated in the mPFC, NAc, Amg and HPC (Figure 2 and Table S2).

In the mPFC, PPS did not significantly affect any of the tested inflammatory markers (Figure 2A and Table S2). In contrast, the levels of IL-1β ($F_{(1,38)} = 17.12$, $p < 0.001$), IL-6 ($F_{(1,38)} = 42.42$, $p < 0.001$) and IBA-1 ($F_{(1,38)} = 67.76$, $p < 0.001$) were significantly increased in H-CSR rats compared with N-CSR rats, independently of PPS exposure (Table S2). In the NAc, PPS significantly impacted the levels of TNF-α ($F_{(1,38)} = 5.24$, $p = 0.0278$). In particular, H-CSR PPS rats showed increased levels of TNF-α compared with H-CSR non-PPS control rats (H-CSR $_{(CTR\ vs\ PPS)}$: TNF-α: $t = 2.730$, $p = 0.019$; Figure 2B and Table S2). Moreover, IL-1β ($F_{(1,38)} = 5.505$, $p = 0.025$), IL-6 ($F_{(1,38)} = 11.40$, $p = 0.002$) and TNF-α ($F_{(1,38)} = 5.955$, $p = 0.019$) expression was upregulated in H-CSR rats compared with N-CSR rats, independently of PPS (Table S2). In the Amg, the expression levels of the tested inflammatory markers were not affected by PPS or different CSRs (Figure 2C and Table S2). In the HPC, PPS exposure significantly impacted the expression levels of TNF-α ($F_{(1,38)} = 11.37$, $p = 0.002$) and IBA-1 ($F_{(1,38)} = 7.18$, $p = 0.011$; Figure 2D and Table S2), independently of CSR. Post hoc analysis revealed that when comparing PPS animals versus the respective control animals, IBA-1 expression was increased in H-CSR rats (H-CSR $_{(CTR\ vs\ PPS)}$: IBA-1: $t = 3.777$, $p = 0.001$), while TNF-α expression was increased in both N-CSR rats (N-CSR $_{(CTR\ vs\ PPS)}$: TNF-α: $t = 2.40$, $p = 0.042$) and H-CSR rats (H-CSR $_{(CTR\ vs\ PPS)}$: TNF-α: $t = 2.385$, $p = 0.044$; Figure 2D and Table S2).

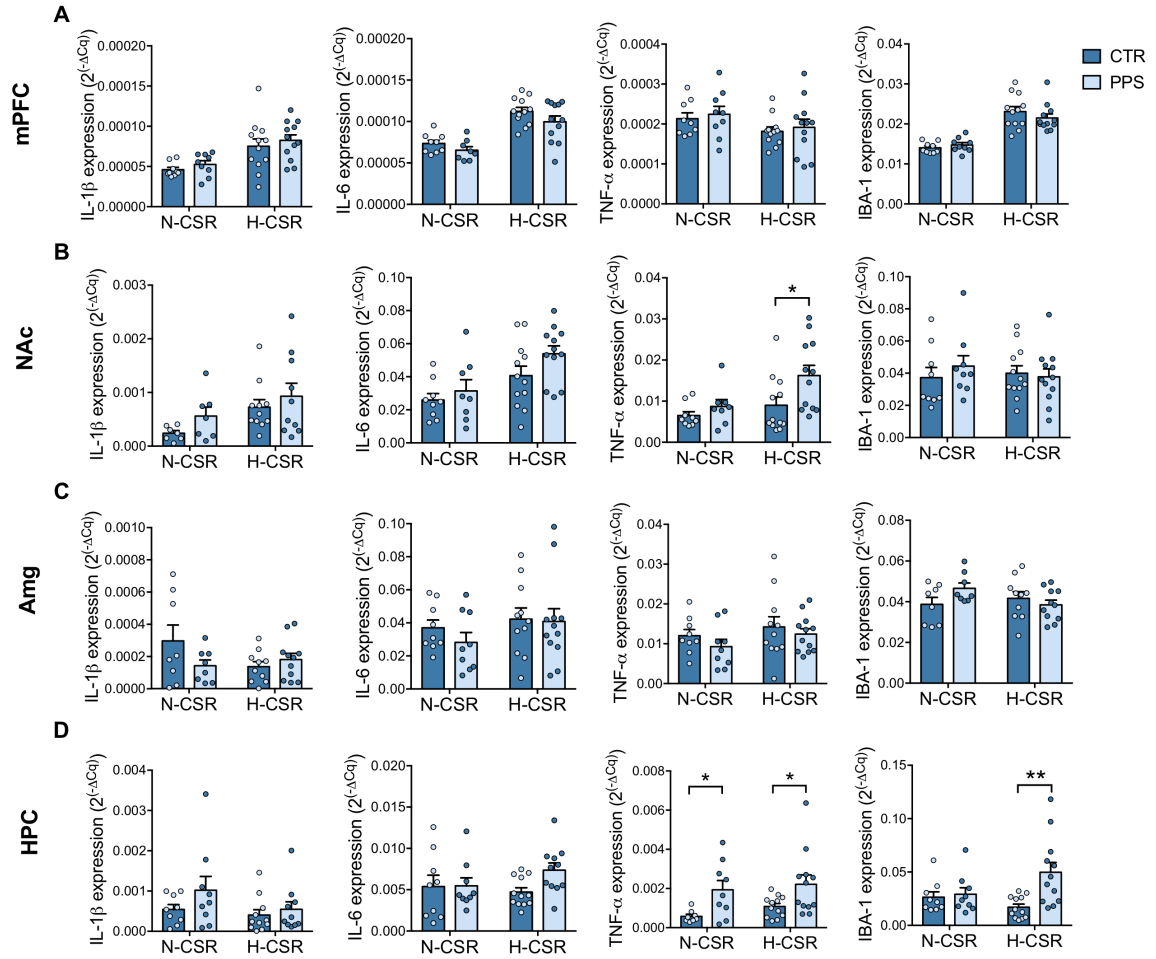


Figure 2. Expression levels of inflammatory markers across different brain regions in adulthood. The mRNA expression levels of IL-1β, IL-6, TNF-α and IBA-1 in the medial prefrontal cortex (mPFC) (A), nucleus accumbens (NAc) (B), amygdala (Amg) (C) and hippocampus (HPC) (D) were evaluated by RT-qPCR using GAPDH and β-actin as internal controls. Relative expression levels were calculated using the quantification cycle (Cq) method, according to the MIQE guidelines, and results are presented as the mean ± SEM. N-CSR = 18 (9 CTR and 9 PPS); H-CSR = 24 (12 CTR and 12 PPS); 2-way ANOVA followed by Sidak's post hoc multiple comparisons test was performed to evaluate significant differences among groups. Statistical differences between CTR and PPS animals are highlighted with asterisks *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

To explore possible correlations between the expression levels of inflammatory molecules and depressive-like behaviours, Pearson correlation analyses were performed. No significant correlations were found between the tested inflammatory markers in the mPFC, NAc and Amg and depressive-like behaviours (Figure S2, S3 and S4). However, in the HPC, TNF-α (but not IL-1β, IL-6 or IBA-1) was positively correlated with the % of time spent floating on the second day of the FST (% time floating vs TNF-α: r = 0.3799, $p = 0.014$; Figure 3), suggesting that increased TNF-α expression levels in the hippocampus might contribute to or be a consequence of depressive-like behaviours.

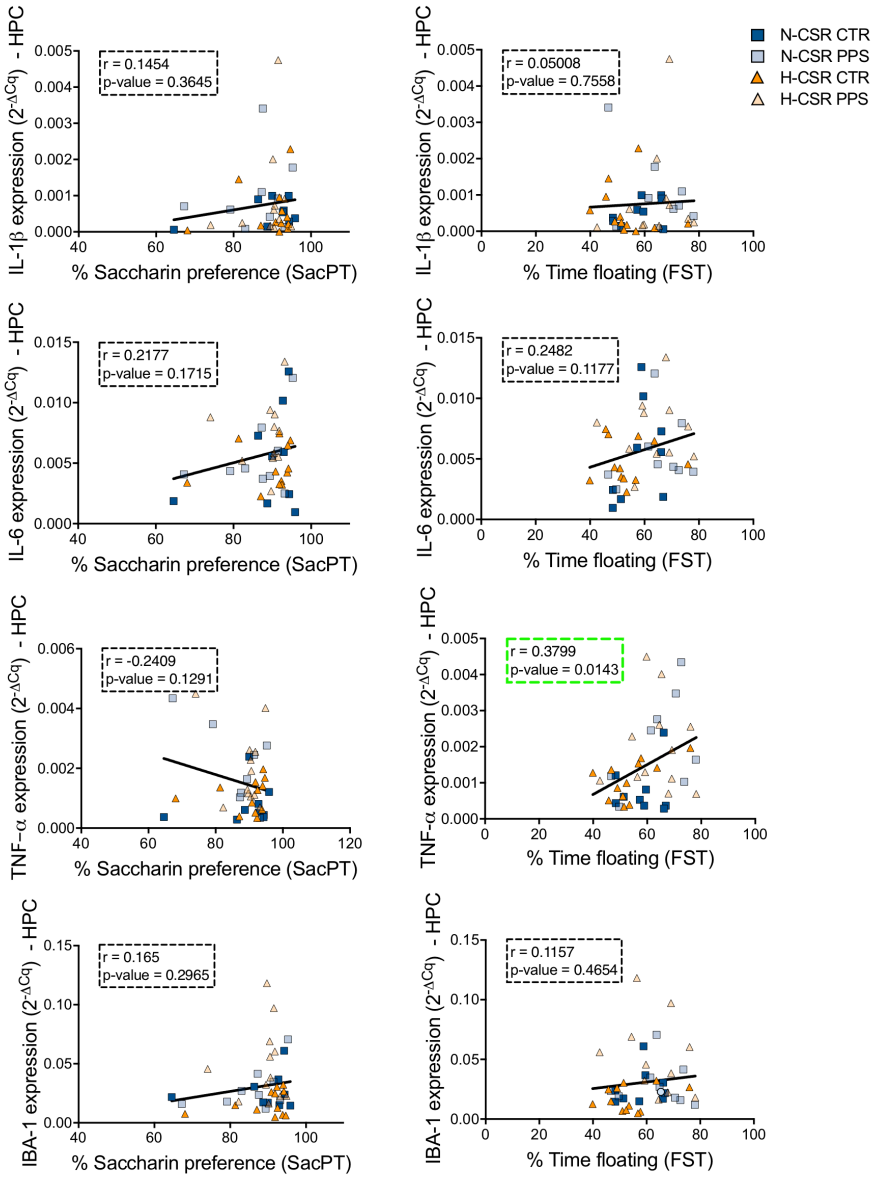


Figure 3. Correlations between the expression levels of inflammatory markers in the hippocampus and depressive-like behaviours. Pearson correlation analyses were performed by comparing the mRNA expression levels of IL-1β, IL-6, TNF-α and IBA-1 in the hippocampus and % saccharin preference (SacPT) or % of time spent floating (FST). Correlations were performed considering all animals (N=42). The coefficient r and *p*-value for each correlation are presented in a box. Statistically significant correlations are highlighted in green (p<0.05).

2.3.3. Peripubertal stress induces microglial activation in the hippocampus of high-CORT stress-responsive rats

Following the detection of increases in TNF-α and IBA-1 in the HPC of adult rats that had been exposed to PPS, particularly H-CSR animals, and considering that hippocampal TNF-α levels were positively correlated with depressive-like behaviour, as measured by the FST (Figure 3), we next investigated the microglial activation status. This evaluation is important for understanding whether hippocampal homeostasis is partially compromised. Typically, activated microglia show an ameboid morphology with an increased soma area, a decreased arborization area and a decreased number of branches (42). Microglial morphology was evaluated by immunofluorescence staining for IBA-1 (a marker of microglial activation) in the CA1, CA3 and dentate gyrus (DG) subregions of the dorsal hippocampus, and 3D morphological analysis was performed with IMARIS software for a more robust analysis (Figure 4A, Figure S5 and S6). On average, more than 20 cells per animal were analysed to evaluate soma volume, arborization volume, convexHull volume, average branch length, max. branch length and total number of branches (Figure 4A-C and Figure S6).

PPS induced alterations in hippocampal microglial morphology in adulthood, particularly in terms of arborization volume ($F_{(1,38)} = 7.045$, $p = 0.012$) and total number of branches ($F_{(1,38)} = 5.186$, $p = 0.029$; Figure 4B and C). Of note, a significant interaction between stress exposure and different CSRs was found in terms of the total number of branches ($F_{(1,38)} = 5.256$, $p = 0.027$). Independent of PPS, different CSRs tended to induce similar alterations in microglial morphology, although the differences did not reach statistical significance (arbo vol: $F_{(1,38)} = 3.806$, $p = 0.058$; branch number: $F_{(1,38)} = 2.857$, $p = 0.099$; Figure 4B and C). Importantly, post hoc analysis revealed that PPS H-CSR rats exhibited significant decreases in arborization volume ($H\text{-}CSR_{(CTR\ vs\ PPS)}$: $t = 3.064$, $p = 0.008$), convexHull volume ($H\text{-}CSR_{(CTR\ vs\ PPS)}$: $t = 2.359$, $p = 0.047$) and total number of branches ($H\text{-}CSR_{(CTR\ vs\ PPS)}$: $t = 3.613$, $p = 0.002$) compared with H-CSR animals in control (non-PPS) conditions, suggesting alterations in PPS H-CSR rat microglial morphology compatible with a higher degree of activation (Figure 4B and C).

Importantly, we found hippocampal IBA-1 expression levels to be negatively correlated with microglial arborization volume (arbo vol vs IBA-1: $r = -0.371$, $p = 0.018$) and with the number of branches (branch number vs IBA-1: $r = -0.372$, $p = 0.018$; Figure 4D). Moreover, although not statistically significant, hippocampal microglial arborization volume tended to be negatively correlated with the % of time spent floating on the second day of the FST (arbo vol vs % time floating: $r = -0.300$, $p = 0.060$; Figure 4E). Overall, these results show increased hippocampal microglial activation in adult H-CSR rats subjected to PPS.

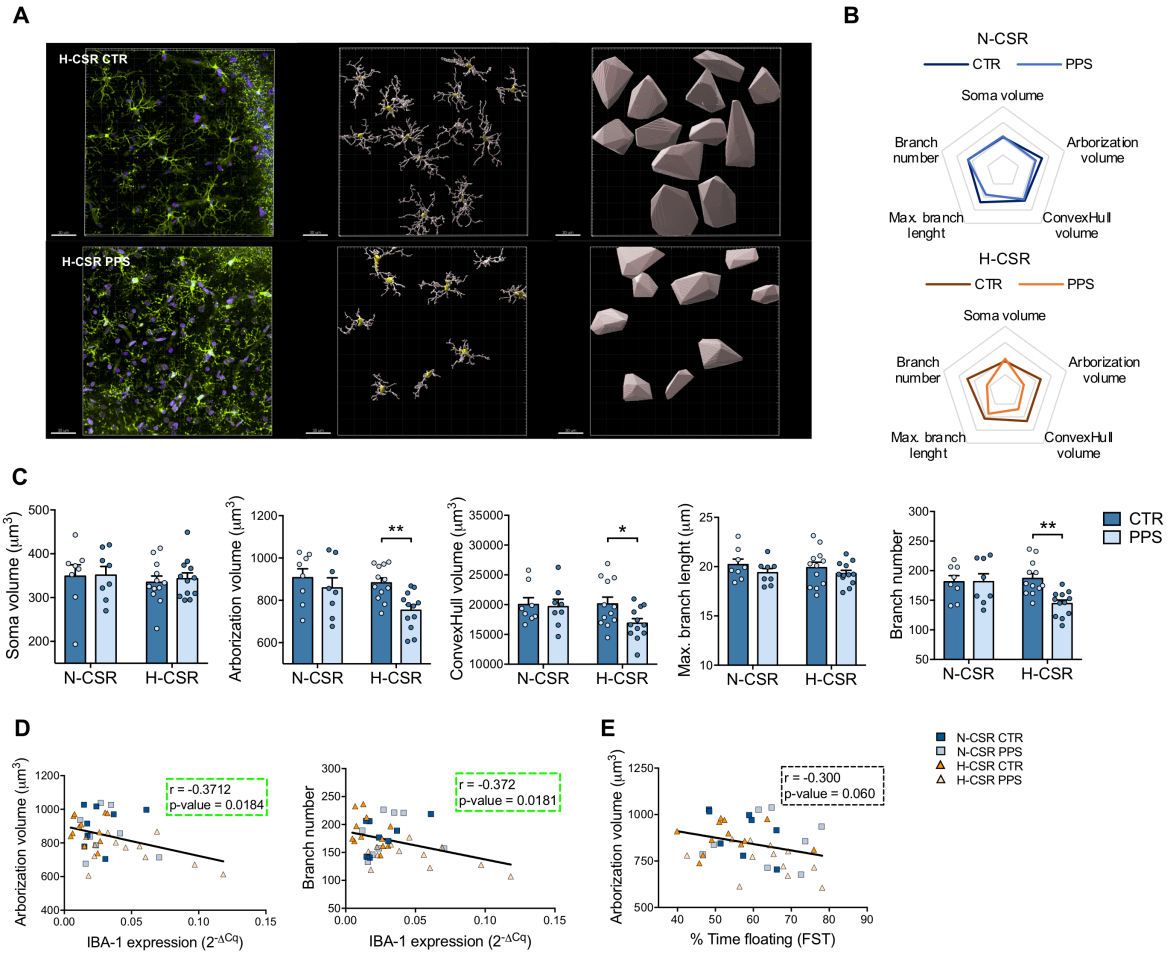


Figure 4. Morphological analysis of hippocampal microglia. To evaluate microglial activation, images of IBA-1-positive microglia in three different subregions of the dorsal hippocampus (CA1, CA3 and DG) were acquired, and microglial morphology analysed. (A) Representative images showing IBA-1-positive microglia in the CA1 subregion in green (left). IMARIS 3D arborization (centre) and IMARIS convexHull (right) visualizations of H-CSR CTR and PPS rats (scale bar: 30 µm). (B) Radar chart containing the average results of the most relevant morphological features analysed. (C) Quantitative analysis of hippocampal microglial morphology. Results represent the average values of the 3 subregions. Each dot represents an animal (N: N-CSR = 18 (9 CTR and 9 PPS); H-CSR = 24 (12 CTR and 12 PPS)). On average, more than 20 cells per rat were analysed for soma volume, arborization volume, convexHull volume, max. branch length and total number of branches. Results are expressed as the mean ± SEM. Two-way ANOVA followed by Sidak's multiple comparisons test, *$p < 0.05$. (D-E) Pearson correlation analyses were performed by comparing significantly altered morphological features (arborization volume and total number of branches) and IBA-1 expression (D) and arborization volume and % of time spent floating (FST) (E). Correlations were performed considering all animals (N=42). The coefficient r and *p*-value for each correlation are presented in a box. Statistically significant correlations are highlighted in green ($p<0.05$).

2.3.4. miR-342 expression in the hippocampus is positively correlated with TNF-α expression, microglial activation and depressive-like behaviours

In addition to cytokines, inflammatory miRNAs (called inflammiRs) are crucial molecules in the regulation of microglial activation (34). This is the case for miR-342, which we previously identified as a key player in TNF-α-driven microglial activation and a potential target for tackling microglia-driven neuroinflammation (35). Considering the morphological changes in the hippocampal microglia of H-CSR adult rats following PPS, we investigated miR-342 expression in the hippocampus. miR-342 expression levels were significantly impacted by stress exposure ($F_{(1,38)}$ = 7.128, p = 0.011; Figure 5A). Post hoc analysis revealed that miR-342 expression levels were significantly increased in H-CSR rats undergoing PPS compared with the respective non-PPS control rats (H-CSR$_{(CTR\ vs\ PPS)}$: t = 2.681, p = 0.021), while no significant differences were found for the N-CSR PPS versus CTR groups (N-CSR$_{(CTR\ vs\ PPS)}$: t = 1.210, p = 0.412; Figure 5A). Interestingly, hippocampal miR-342 expression was positively correlated with TNF-α expression (miR-342 vs TNF-α: r = 0.400; p = 0.009; Figure 5B) and negatively correlated with microglial arborization volume (miR-342 vs arbo vol: r = -0.351; p = 0.026; Figure 5C).

Subsequently, the correlation between hippocampal miR-342 expression and depressive-like behaviours revealed a significant positive correlation between miR-342 levels and the percentage of time spent floating on the second day of the FST (miR-342 vs % time floating: r = 0.479; p = 0.0013; Figure 5D) and a tendency toward a negative correlation with saccharin preference (miR-342 vs % sac pref: r = -0.288; p = 0.063; Figure 5D). These results suggest that hippocampal miR-342 expression may be a factor in microglial activation and depression susceptibility.

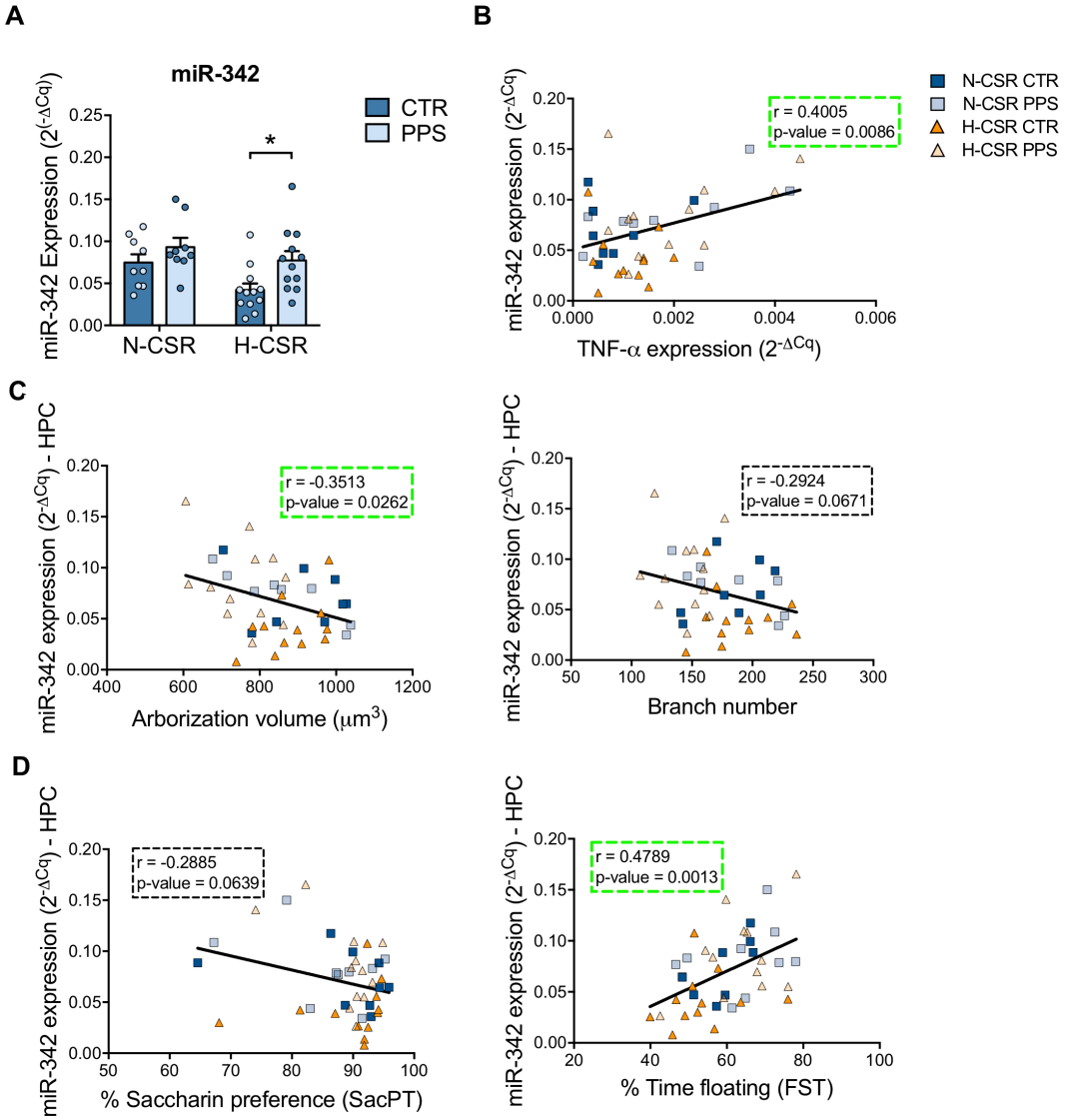


Figure 5. miR-342 expression analysis in the hippocampus. (A) miR-342 expression levels in the hippocampus were evaluated by RT-qPCR using U6 snRNA as an internal control. Relative expression levels were calculated using the quantification cycle (Cq) method, according to the MIQE guidelines, and results are presented as the mean ± SEM. N: N-CSR = 18 (9 CTR and 9 PPS); H-CSR = 24 (12 CTR and 12 PPS). Two-way ANOVA followed by Sidak's multiple comparisons test, **p* < 0.05. (B-D) Pearson correlation analyses were performed to evaluate possible correlations between hippocampal miR-342 expression and TNF-α expression (B), microglial activation/altered morphological features (C) and depressive-like behaviours (D). Correlations were performed considering all animals (N=42). The coefficient r and *p*-value for each correlation are presented in a box. Statistically significant correlations are highlighted in green (p<0.05).

2.4. Discussion

The “inflammation-related depression” hypothesis has been under debate, and recent findings increasingly support a link between inflammation and depression. First, it has been reported that a subgroup of depression patients exhibits increased levels of peripheral inflammatory markers (9, 43). Second, proinflammatory cytokines have been found to be elevated in the cerebrospinal fluid of a segment of depression patients (44). Third, while studies regarding the anti-inflammatory properties of traditional antidepressants are contradictory (45-48), inflammation is more aberrant in treatment non-responders (49, 50), with significant associations between the number of failed treatment trials and increased levels of TNF-α, IL-6 and C-reactive protein (50). These findings have identified new challenges in the discovery of inflammatory mediators that can serve as biomarkers and/or alternative targets for the treatment of inflammation-related depression.

Early-life stress exposure and constitutive differences in glucocorticoid responsiveness to stressors can influence the risk of depression (51-53), but their effect on inflammatory mediators in the brain remain largely unexplored. We investigated how these two factors influence depressive-like behaviours, neuroinflammation and their neurobiological correlates. Although PPS alone was sufficient to induce reduced sociability in male rats, independent of CORT stress responsiveness, the results showed that only those with a lower degree of adaptation of their CORT responses (H-CSR group) exhibited increased anhedonia and passive coping responses in adulthood. This suggests that increased CORT levels enhance the susceptibility of animals to late effects of stress and, consequently, the exhibition of depressive-like behaviours in adulthood. Although a chronic increase in CORT was previously reported to be sufficient to induce depression-like behaviours in rats (54), we found that high-CSR alone, without priming by stressful events, was not sufficient to induce depressive-like behaviours in male rats. Nonetheless, a limitation of this study is the use of males only, while sex-specific mechanisms have been shown to regulate response to stress and the development of depression outcomes (55). A question to address in the future is whether sex differences influence the long-term effect of PPS in terms of brain inflammatory markers.

Following our hypothesis that PPS in combination with H-CSR might contribute to increased inflammation and depression-like behaviours, we evaluated the expression of inflammatory markers across different brain regions in adulthood. We found that animals with high-CORT stress responsiveness exhibited increased levels of IL-1β, IL-6 and IBA-1 in the mPFC and IL-1β, IL-6 and TNF-α in the NAc. With the exception of TNF-α in the NAc, all these inflammatory markers were altered independently of PPS, suggesting that their levels in these regions may not be associated with depressive-like behaviours but rather with CORT regulation. In contrast, TNF-α expression in the NAc was increased in high-CSR rats that

underwent PPS (i.e., rats exhibiting depressive-like behaviours). However, these levels in the NAc were not statistically correlated with either the % of saccharin preference or % of floating time in the FST. Interestingly, TNF-α expression was also increased in the hippocampus of both N-CSR and H-CSR rats submitted to PPS, and most importantly, hippocampal TNF-α expression was positively correlated with the worsening of depressive-like behaviours (as shown by FST). In fact, several clinical trials have provided evidence on the efficacy of different anti-TNF-α drugs in depressive symptoms/disorder (56). Monoclonal antibody drugs against TNF-α, such as infliximab and adalimumab, were shown to be effective in improving mood in treatment-resistant depression patients (57), as well as in Chron's disease and psoriasis patients (58, 59). On the other hand, etanercept, a TNF-α receptor blocker, was also shown to reduce secondary depressive symptoms to some extent in patients suffering from psoriasis (60, 61).

Despite having an important role in brain development (62), a chronic increase in TNF-α levels is known to have deleterious effects on brain cells and neurocircuits. First, TNF-α is known to induce indoleamine 2,3 dioxygenase (IDO)-mediated tryptophan degradation through the kynurenine pathway, which generates neuroactive metabolites, including kynurenic acid and quinolinic acid, ultimately influencing serotonergic neurotransmission (63). Second, TNF-α can potentiate glutamate-mediated excitoneurotoxicity indirectly by inhibiting glutamate transport on astrocytes and directly by stimulating extensive microglial and/or astrocyte glutamate release in an autocrine manner (12, 13, 64). Indeed, in response to their dynamic surrounding environment, microglia display a remarkable degree of phenotypic plasticity, in terms of both morphology and molecular markers (65). While microglial reactivity may initially contribute to the restoration of tissue homeostasis, persistent inflammation that overstimulates microglia leads to a sequence of events that compromise neuronal survival (66).

In fact, we found that PPS produced late effects on hippocampal microglia, particularly in animals with high-CSR (i.e., animals exhibiting depressive-like behaviours), with increased hippocampal IBA-1 expression and morphological alterations compatible with a higher degree of activation. In line with our findings, Gong *et al.* reported that early social isolation in mice induced depressive-like behaviours and loss and dystrophy of hippocampal microglia in adulthood, caused by increased activation of these cells (67). Nonetheless, since separate hemispheres were used to perform gene expression and microglia morphological analysis, we do not exclude any laterality effect. Additionally, it is possible that the dysregulation of microglial activation and cytokine expression levels may also be occurring in other brain regions, as described by others (68). However, according to the expression levels of the inflammatory markers tested over distinct brain regions, alterations in TNF-α and IBA-1 expression were more evident in the hippocampus. In agreement, multiple reports focusing

on the late effects of early-life stress in terms of depressive-like behaviours highlight major alterations in the hippocampus compared with other regions of the brain (69).

Identifying specific molecules underlying cytokine expression, microglial dysregulation and depression symptoms is of interest for the development of novel targeted therapeutic drugs. Since the first demonstration of their involvement in human diseases in 2002 (70), miRNAs have emerged as therapeutic targets in distinct pathologies due to their capacity to regulate protein levels, including those of inflammatory markers, and influence the course of disease, patient response to treatment and clinical outcome (71). However, few studies have referred to the late effects of early-life stress on depression and miRNA expression. Among studied miRNAs, miR-124a and miR-18a were shown to be upregulated in the PFC and HPC in adulthood and were associated with depressive-like behaviours (72), while miR-146a overexpression was shown to improve depressive-like behaviours in mice by inhibiting microglial activation (73) and to protect against cognitive decline-induced surgical trauma by suppressing hippocampal neuroinflammation (74). In a previous *in vitro* study, we identified miR-342 as a potential target for resolving neuroinflammation (35). In particular, miR-342 was found to be upregulated in microglia primed with TNF-α, and its overexpression was shown to activate the NF-κB pathway, leading to increased secretion of TNF-α, IL-1β and nitrites and drastically affecting neuron viability (35). Herein, we associated hippocampal miR-342 expression with depressive-like behaviours for the first time. Strikingly, we found hippocampal miR-342 to be positively correlated with TNF-α expression and microglial activation. These results not only support the previously established hypothesis of strong interplay between miR-342 and TNF-α in potentiating microglial activation but also identify miR-342 as a novel potential therapeutic target for microglial activation in the hippocampus and for inflammation-related depression. Moreover, since inflammatory mediators in the hippocampus were specifically correlated with forced swim performance, an alternative line of research is to address is how hippocampal TNF-α, microglia and miR-342 mediate the programming effects of PPS on memory performance in adulthood (75).

Of note, preclinical studies in Alzheimer's disease (AD) and Parkinson's disease (PD) using miR-342-3p antagomir and anti-miR-342-3p constructs, respectively, have shown promising results (76, 77). Intrahippocampal miR-342 inhibition was shown to reduce β-amyloid plaques and ameliorate learning and memory in 3xTg-AD mice (76), a widely used murine model of pathological or behavioural abnormalities of AD, while suppression of miR-342 improved the expression of glutamate transporter, promoted dopaminergic neuron proliferation and suppressed apoptosis through the Wnt signalling pathway in mice with PD (77). Due to the interconnectivity and overlap of many neurocircuits and mechanisms between neurodegenerative and psychiatric diseases, particularly in terms of neuroinflammation and

microglial morphological and functional changes, we suggest miR-342 as a potential candidate target mediating inflammation-related depression.

In conclusion, we show that stressful early-life events, combined with high-CORT stress responsiveness, potentiate the development of depression-like behaviours and neuro-immunological alterations in adulthood, particularly increased microglial activation, TNF-α and miR-342 expression levels in the hippocampus. The potential of miR-342 as a therapeutic target in inflammation-related depression should be further investigated.

Acknowledgments

Authors would like to thank Dr. Arne Seitz and Dr. Romain Guiet from BioImaging and Optics Platform (BIOP, EPFL) for their valuable help in performing microglia morphological analysis. This project has been supported by grants from the Swiss National Science Foundation (SNSF) [NCCR Synapsy: 51NF40-158776 and -185897] and ERA-NET (Biostress; SNSF No 31NE30_189061). JPB was supported by FCT- Fundação para a Ciência e a Tecnologia, through BiotechHealth PhD program fellowship (PD/BD/135490/2018). SM was supported by NCCR Synapsy grant. MM was supported by an internal grant from the Donders Centre for Medical Neurosciences of the Radboudumc, and a personal travel grant from FENS. MIA was also supported by FCT (CEECINST/00091/2018/CP1500/CT0011). The funding sources had no additional role in study design, in the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

References

1. Krishnan V, Nestler EJ. The molecular neurobiology of depression. Nature. 2008;455(7215):894-902.

2. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nat Rev Immunol. 2016;16(1):22-34.

3. Miller AH. Beyond depression: the expanding role of inflammation in psychiatric disorders. World Psychiatry. 2020;19(1):108-9.

4. O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. Journal of psychiatric research. 2007;41(3-4):326-31.

5. Fitzgerald P, O'Brien SM, Scully P, Rijkers K, Scott LV, Dinan TG. Cutaneous glucocorticoid receptor sensitivity and pro-inflammatory cytokine levels in antidepressant-resistant depression. Psychological medicine. 2006;36(1):37-43.

6. Harrison NA, Brydon L, Walker C, Gray MA, Steptoe A, Critchley HD. Inflammation causes mood changes through alterations in subgenual cingulate activity and mesolimbic connectivity. Biological psychiatry. 2009;66(5):407-14.

7. Lanquillon S, Krieg JC, Bening-Abu-Shach U, Vedder H. Cytokine production and treatment response in major depressive disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2000;22(4):370-9.

8. Sluzewska A, Sobieska M, Rybakowski JK. Changes in acute-phase proteins during lithium potentiation of antidepressants in refractory depression. Neuropsychobiology. 1997;35(3):123-7.

9. Brás JP, Pinto S, Almeida MI, Prata J, von Doellinger O, Coelho R, et al. Peripheral Biomarkers of Inflammation in Depression: Evidence from Animal Models and Clinical Studies. In: Kobeissy FH, editor. Psychiatric Disorders: Methods and Protocols: Springer; 2019. p. 467-92.

10. Dheen ST, Kaur C, Ling EA. Microglial activation and its implications in the brain diseases. Current medicinal chemistry. 2007;14(11):1189-97.

11. Yirmiya R, Rimmerman N, Reshef R. Depression as a microglial disease. Trends in neurosciences. 2015;38(10):637-58.

12. Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, et al. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci. 2001;4(7):702-10.

13. Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, et al. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. The Journal of biological chemistry. 2006;281(30):21362-8.

14. Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nature reviews Neuroscience. 2007;8.

15. Kuno R, Wang J, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. Autocrine activation of microglia by tumor necrosis factor-alpha. J Neuroimmunol. 2005;162(1-2):89-96.

16. Remus JL, Dantzer R. Inflammation Models of Depression in Rodents: Relevance to Psychotropic Drug Discovery. The international journal of neuropsychopharmacology. 2016;19(9).

17. Lasselin J, Schedlowski M, Karshikoff B, Engler H, Lekander M, Konsman JP. Comparison of bacterial lipopolysaccharide-induced sickness behavior in rodents and humans: Relevance for symptoms of anxiety and depression. Neuroscience & Biobehavioral Reviews. 2020;115:15-24.

18. Papilloud A, Guillot de Suduiraut I, Zanoletti O, Grosse J, Sandi C. Peripubertal stress increases play fighting at adolescence and modulates nucleus accumbens CB1 receptor

expression and mitochondrial function in the amygdala. Translational psychiatry. 2018;8(1):156.

19. Walker SE, Zanoletti O, Guillot de Suduiraut I, Sandi C. Constitutive differences in glucocorticoid responsiveness to stress are related to variation in aggression and anxiety-related behaviors. Psychoneuroendocrinology. 2017;84:1-10.

20. Hohmann CF, Odebode G, Naidu L, Koban M. Early Life Stress Alters Adult Inflammatory Responses in a Mouse Model for Depression. Ann Psychiatry Ment Health. 2017;5(2):1095.

21. Miller AH. Inflammation Versus Glucocorticoids as Purveyors of Pathology during Stress: Have We Reached the Tipping Point? Biological psychiatry. 2008;64(4):263-5.

22. Heim C, Binder EB. Current research trends in early life stress and depression: review of human studies on sensitive periods, gene-environment interactions, and epigenetics. Experimental neurology. 2012;233(1):102-11.

23. Negele A, Kaufhold J, Kallenbach L, Leuzinger-Bohleber M. Childhood Trauma and Its Relation to Chronic Depression in Adulthood. Depress Res Treat. 2015;2015:650804-.

24. Marquez C, Poirier GL, Cordero MI, Larsen MH, Groner A, Marquis J, et al. Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression. Translational psychiatry. 2013;3:e216.

25. Reader BF, Jarrett BL, McKim DB, Wohleb ES, Godbout JP, Sheridan JF. Peripheral and Central Effects of Repeated Social Defeat Stress: Monocyte Trafficking, Microglial Activation, and Anxiety. Neuroscience. 2015;289:429-42.

26. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nature Reviews Neuroscience. 2009;10(6):397-409.

27. Sorrells SF, Sapolsky RM. An inflammatory review of glucocorticoid actions in the CNS. Brain, behavior, and immunity. 2007;21(3):259-72.

28. Iob E, Kirschbaum C, Steptoe A. Persistent depressive symptoms, HPA-axis hyperactivity, and inflammation: the role of cognitive-affective and somatic symptoms. Molecular psychiatry. 2020;25(5):1130-40.

29. Silverman MN, Sternberg EM. Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. Annals of the New York Academy of Sciences. 2012;1261:55-63.

30. Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM. The Stressed CNS: When Glucocorticoids Aggravate Inflammation. Neuron. 2009;64(1):33-9.

31. Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutation research. 2011;717(1-2):1-8.

32. Wakabayashi T, Hidaka R, Fujimaki S, Asashima M, Kuwabara T. MicroRNAs and epigenetics in adult neurogenesis. Advances in genetics. 2014;86:27-44.

33. Issler O, Haramati S, Paul ED, Maeno H, Navon I, Zwang R, et al. MicroRNA 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity. Neuron. 2014;83(2):344-60.

34. Brites D, Fernandes A. Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation. Front Cell Neurosci. 2015;9:476.

35. Brás JP, Bravo J, Freitas J, Barbosa MA, Santos SG, Summavielle T, et al. TNF-alpha-induced microglia activation requires miR-342: impact on NF-κB signaling and neurotoxicity. Cell Death & Disease. 2020;11(6):415.

36. Larsen MH, Mikkelsen JD, Hay-Schmidt A, Sandi C. Regulation of brain-derived neurotrophic factor (BDNF) in the chronic unpredictable stress rat model and the effects of chronic antidepressant treatment. Journal of psychiatric research. 2010;44(13):808-16.

37. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.

38. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nature Methods. 2012;9(7):676-82.

39. Ollion J, Cochennec J, Loll F, Escudé C, Boudier T. TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization. Bioinformatics. 2013;29(14):1840-1.

40. Walker SE, Sandi C. Long-term programing of psychopathology-like behaviors in male rats by peripubertal stress depends on individual's glucocorticoid responsiveness to stress. Stress (Amsterdam, Netherlands). 2018;21(5):433-42.

41. Inostroza M, Cid E, Menendez de la Prida L, Sandi C. Different Emotional Disturbances in Two Experimental Models of Temporal Lobe Epilepsy in Rats. PloS one. 2012;7(6):e38959.

42. Davis BM, Salinas-Navarro M, Cordeiro MF, Moons L, De Groef L. Characterizing microglia activation: a spatial statistics approach to maximize information extraction. Scientific Reports. 2017;7(1):1576.

43. Osimo EF, Pillinger T, Rodriguez IM, Khandaker GM, Pariante CM, Howes OD. Inflammatory markers in depression: A meta-analysis of mean differences and variability in 5,166 patients and 5,083 controls. Brain, behavior, and immunity. 2020;87:901-9.

44. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A meta-analysis of cytokines in major depression. Biological psychiatry. 2010;67(5):446-57.

45. Hiles SA, Baker AL, de Malmanche T, Attia J. Interleukin-6, C-reactive protein and interleukin-10 after antidepressant treatment in people with depression: a meta-analysis. Psychological medicine. 2012;42(10):2015-26.

46. Sluzewska A, Rybakowski JK, Laciak M, Mackiewicz A, Sobieska M, Wiktorowicz K. Interleukin-6 serum levels in depressed patients before and after treatment with fluoxetine. Annals of the New York Academy of Sciences. 1995;762:474-6.

47. Jazayeri S, Keshavarz SA, Tehrani-Doost M, Djalali M, Hosseini M, Amini H, et al. Effects of eicosapentaenoic acid and fluoxetine on plasma cortisol, serum interleukin-1beta and interleukin-6 concentrations in patients with major depressive disorder. Psychiatry research. 2010;178(1):112-5.

48. Kubera M, Kenis G, Bosmans E, Kajta M, Basta-Kaim A, Scharpe S, et al. Stimulatory effect of antidepressants on the production of IL-6. International immunopharmacology. 2004;4(2):185-92.

49. Strawbridge R, Arnone D, Danese A, Papadopoulos A, Herane Vives A, Cleare AJ. Inflammation and clinical response to treatment in depression: A meta-analysis. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2015;25(10):1532-43.

50. Haroon E, Daguanno AW, Woolwine BJ, Goldsmith DR, Baer WM, Wommack EC, et al. Antidepressant treatment resistance is associated with increased inflammatory markers in patients with major depressive disorder. Psychoneuroendocrinology. 2018;95:43-9.

51. Bunea IM, Szentágotai-Tătar A, Miu AC. Early-life adversity and cortisol response to social stress: a meta-analysis. Translational psychiatry. 2017;7(12):1274.

52. Taylor SE. Mechanisms linking early life stress to adult health outcomes. Proceedings of the National Academy of Sciences. 2010;107(19):8507-12.

53. Juruena MF. Early-life stress and HPA axis trigger recurrent adulthood depression. Epilepsy & Behavior. 2014;38:148-59.

54. Johnson SA, Fournier NM, Kalynchuk LE. Effect of different doses of corticosterone on depression-like behavior and HPA axis responses to a novel stressor. Behavioural brain research. 2006;168(2):280-8.

55. Goodwill HL, Manzano-Nieves G, Gallo M, Lee H-I, Oyerinde E, Serre T, et al. Early life stress leads to sex differences in development of depressive-like outcomes in a mouse model. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2019;44(4):711-20.

56. Kappelmann N, Lewis G, Dantzer R, Jones PB, Khandaker GM. Antidepressant activity of anti-cytokine treatment: a systematic review and meta-analysis of clinical trials of chronic inflammatory conditions. Molecular psychiatry. 2018;23(2):335-43.

57. Raison CL, Rutherford RE, Woolwine BJ, et al. A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: The role of baseline inflammatory biomarkers. JAMA Psychiatry. 2013;70(1):31-41.

58. Loftus EV, Feagan BG, Colombel JF, Rubin DT, Wu EQ, Yu AP, et al. Effects of adalimumab maintenance therapy on health-related quality of life of patients with Crohn's disease: patient-reported outcomes of the CHARM trial. The American journal of gastroenterology. 2008;103(12):3132-41.

59. Menter A, Augustin M, Signorovitch J, Yu AP, Wu EQ, Gupta SR, et al. The effect of adalimumab on reducing depression symptoms in patients with moderate to severe psoriasis: a randomized clinical trial. Journal of the American Academy of Dermatology. 2010;62(5):812-8.

60. Tyring S, Gottlieb A, Papp K, Gordon K, Leonardi C, Wang A, et al. Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet (London, England). 2006;367(9504):29-35.

61. Tyring S, Bagel J, Lynde C, Klekotka P, Thompson EH, Gandra SR, et al. Patient-reported outcomes in moderate-to-severe plaque psoriasis with scalp involvement: results from a randomized, double-blind, placebo-controlled study of etanercept. Journal of the European Academy of Dermatology and Venereology : JEADV. 2013;27(1):125-8.

62. Golan H, Levav T, Mendelsohn A, Huleihel M. Involvement of tumor necrosis factor alpha in hippocampal development and function. Cerebral cortex (New York, NY : 1991). 2004;14(1):97-105.

63. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience. 2008;9(1):46-56.

64. Olmos G, Llado J. Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. Mediators of inflammation. 2014;2014:12.

65. Gomez-Nicola D, Perry VH. Microglial Dynamics and Role in the Healthy and Diseased Brain: A Paradigm of Functional Plasticity. The Neuroscientist. 2014;21(2):169-84.

66. Tang Y, Le W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Molecular Neurobiology. 2016;53(2):1181-94.

67. Gong Y, Tong L, Yang R, Hu W, Xu X, Wang W, et al. Dynamic changes in hippocampal microglia contribute to depressive-like behavior induced by early social isolation. Neuropharmacology. 2018;135:223-33.

68. Pan Y, Chen XY, Zhang QY, Kong LD. Microglial NLRP3 inflammasome activation mediates IL-1β-related inflammation in prefrontal cortex of depressive rats. Brain, behavior, and immunity. 2014;41:90-100.

69. Catale C, Gironda S, Lo Iacono L, Carola V. Microglial Function in the Effects of Early-Life Stress on Brain and Behavioral Development. J Clin Med. 2020;9(2):468.

70. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proceedings of the National Academy of Sciences. 2002;99(24):15524-9.

71. Almeida MI, Reis RM, Calin GA. Decoy activity through microRNAs: the therapeutic implications. Expert opinion on biological therapy. 2012;12(9):1153-9.

72. Xu J, Wang R, Liu Y, Wang W, Liu D, Jiang H, et al. Short- and long-term alterations of FKBP5-GR and specific microRNAs in the prefrontal cortex and hippocampus of male rats induced by adolescent stress contribute to depression susceptibility. Psychoneuroendocrinology. 2019;101:204-15.

73. Liu CP, Zhong M, Sun JX, He J, Gao Y, Qin FX. miR-146a reduces depressive behavior by inhibiting microglial activation. Molecular medicine reports. 2021;23(6).

74. Chen L, Dong R, Lu Y, Zhou Y, Li K, Zhang Z, et al. MicroRNA-146a protects against cognitive decline induced by surgical trauma by suppressing hippocampal neuroinflammation in mice. Brain, behavior, and immunity. 2019;78:188-201.

75. Tzanoulinou S, Gantelet E, Sandi C, Márquez C. Programming effects of peripubertal stress on spatial learning. Neurobiology of Stress. 2020;13:100282.

76. Fu Y, Hu X, Zheng C, Sun G, Xu J, Luo S, et al. Intrahippocampal miR-342-3p inhibition reduces β-amyloid plaques and ameliorates learning and memory in Alzheimer's disease. Metabolic brain disease. 2019;34(5):1355-63.

77. Wu DM, Wang S, Wen X, Han XR, Wang YJ, Shen M, et al. Suppression of microRNA-342-3p increases glutamate transporters and prevents dopaminergic neuron loss through activating the Wnt signaling pathway via p21-activated kinase 1 in mice with Parkinson's disease. Journal of cellular physiology. 2019;234(6):9033-44.

Supplementary Information

Stress-induced depressive-like behaviour in male rats is associated with microglial activation and inflammation dysregulation in the hippocampus in adulthood

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Table S1

Table S1. Oligonucleotides sequences for RT-qPCR. Oligonucleotides used to amplify rat mRNAs encoding inflammatory markers and reference genes, based on GenBank sequences. Abbreviations: Iba1, ionized calcium-binding adapter molecule 1; Il1b, interleukin 1 beta; Il6, interleukin 6; Tnf, tumour necrosis factor; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; B-actin, beta-actin.

Gene	Accession Number	Forward (5'-3')	Reverse (5'-3')
Rat *Iba1*	NM_017196	GCCTCATCGTCATCTCCCCA	AGGAAGTGCTTGTTGATCCCA
Rat *Il1b*	NM_031512	CACCTTCTTTTCCTTCATCTTTG	GTCGTTGCTTGTCTCTCCTTGTA
Rat *Il6*	NM_012589	CATATGTTCTCAGGGAGATCTTGGA	CAGTGCATCATCGCTGTTCA
Rat *Tnf*	NM_012675	CCCAGACCCTCACACTCAGAT	TTGTCCCTTGAAGAGAACCTG
Rat *Gapdh*	NM_017008	CCCCCAATGTATCCGTTGTG	TAGCCCAGGATGCCCTTTAGT
Rat *B-actin*	NM_031144	AGAAGAGCTATGAGCTGCCTGACG	TACTTGCGCTCAGGAGGAGCAATG

Table S2

Table S2. Expression levels of inflammatory markers across different brain regions in adulthood. The mRNA expression levels of IL-1β, IL-6, TNF-α and IBA-1 in the mPFC, NAc, Amg and HPC were evaluated by RT-qPCR using GAPDH and β-actin as internal controls. Relative expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines, and results are presented as mean ± SEM. N: N-CSR = 18 (9 CTR and 9 PPS); H-CSR = 24 (12 CTR and 12 PPS); 2-way ANOVA test was performed to evaluate the impact of different CSRs and stress exposure in the expression levels of the inflammatory markers. Differences between corresponding CTR and PPS groups were evaluated by Sidak's post-hoc multiple comparisons. Where a significant difference was found between CTR and PPS groups, gene names are shown in bold (n.s.: non-significant; *$p < 0.05$; **p<0.01; ***p<0.001).

Brain Region	Gene	N-CSR				H-CSR				2-way ANOVA			
		CTR (n=9)		PPS (n=9)		CTR (n=12)		PPS (n=12)		Row factor (N-CSRvsH-CSR)		Post-hoc Comparison	
		mean	SEM	mean	SEM	mean	SEM	mean	SEM	F	*p*-value	N-CSR: CTRvsPPS	H-CSR: CTRvsPPS
mPFC	*Il1b*	0.000046	0.000003	0.000053	0.000005	0.000075	0.000009	0.000082	0.000007	17.12	***	n.s.	n.s.
	Il6	0.000074	0.000004	0.000065	0.000004	0.000112	0.000005	0.000100	0.000007	42.32	***	n.s.	n.s.
	Tnf	0.000213	0.000015	0.000224	0.000020	0.000182	0.000011	0.000192	0.000020	3.56	n.s.	n.s.	n.s.
	Iba1	0.014040	0.000453	0.014730	0.000594	0.023110	0.001216	0.021510	0.000980	67.76	***	n.s.	n.s.
NAc	*Il1b*	0.000240	0.000050	0.000560	0.000168	0.000727	0.000139	0.000930	0.000242	5.51	*	n.s.	n.s.
	Il6	0.025950	0.003921	0.031440	0.006846	0.040660	0.005745	0.053930	0.004819	11.40	**	n.s.	n.s.
	Tnf	0.006513	0.000943	0.008705	0.001661	0.009014	0.001989	0.016230	0.002476	5.96	*	n.s.	*
	Iba1	0.037230	0.006375	0.044400	0.006552	0.040000	0.004609	0.037750	0.004921	0.12	n.s.	n.s.	n.s.
Amg	*Il1b*	0.000297	0.000098	0.000142	0.000036	0.000137	0.000031	0.000181	0.000039	1.28	n.s.	n.s.	n.s.
	Il6	0.037020	0.004712	0.028200	0.005969	0.042220	0.006793	0.040810	0.007785	1.72	n.s.	n.s.	n.s.
	Tnf	0.012050	0.001540	0.009273	0.001821	0.014230	0.002587	0.012430	0.001346	1.92	n.s.	n.s.	n.s.
	Iba1	0.038760	0.003382	0.046600	0.002575	0.041680	0.003269	0.038450	0.002361	0.79	n.s.	n.s.	n.s.
HPC	*Il1b*	0.000545	0.000120	0.001020	0.000345	0.000405	0.000133	0.000549	0.000184	2.16	n.s.	n.s.	n.s.
	Il6	0.005385	0.001359	0.005464	0.000974	0.004722	0.000513	0.007372	0.000862	0.45	n.s.	n.s.	n.s.
	Tnf	0.000579	0.000108	0.001936	0.000475	0.001082	0.000155	0.001994	0.000362	1.12	n.s.	*	*
	Iba1	0.026440	0.004994	0.029170	0.006052	0.017010	0.002968	0.049720	0.009336	0.71	n.s.	n.s.	**

Figure S1

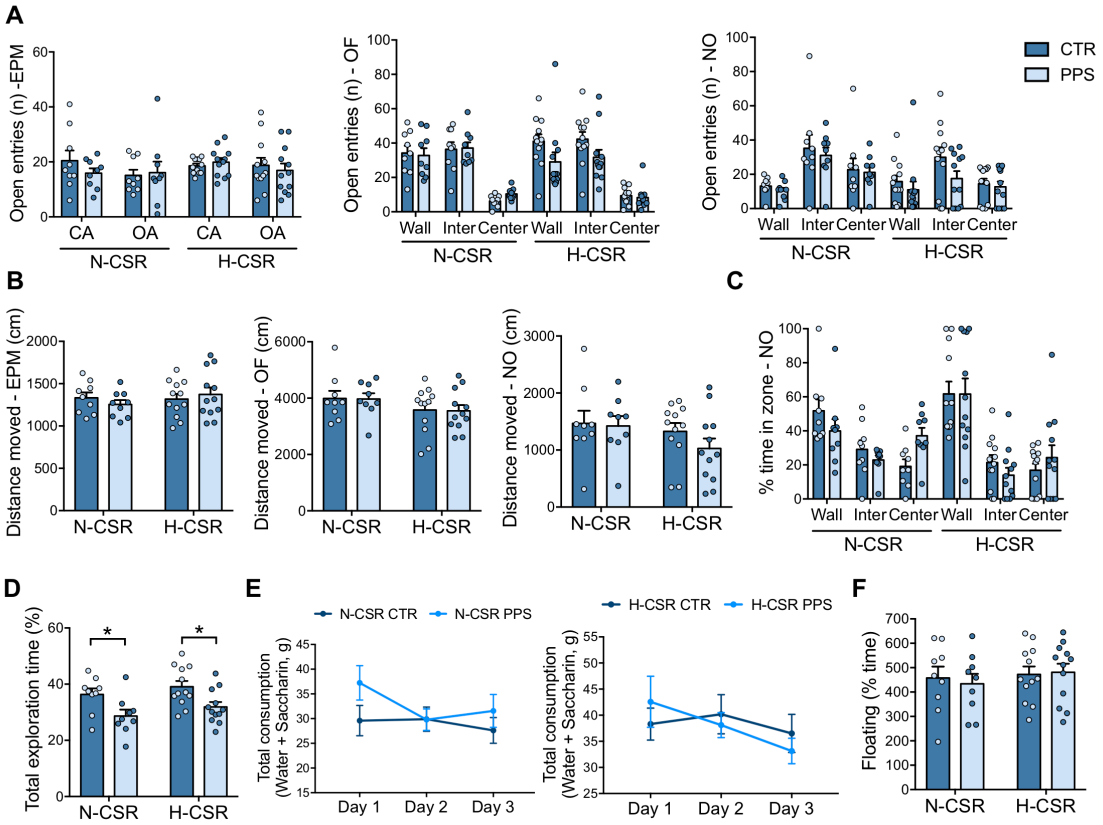


Figure S1. Supplementary behavioural results. Number of open entries in zone (A) and total distance moved (B) in the EPM and OF/NO were calculated using EthoVision 11. After the OF test, a NO was introduced in the centre and the behavior observed for extra 5 min. % time spent in zone and was calculated using EthoVision 11 (C). In the SocPT, total exploration time was scored using Observer X11 (D). In the SacPT, total consumption (water + saccharin) was calculated (E). In the FST, two trials were performed and recorded. The graph represents the % time the animal spent floating in the first trial, lasting 15 min (F). N: N-CSR = 18 (9 CTR and 9 PPS); H-CSR = 24 (12 CTR and 12 PPS). Results are expressed as mean ± SEM. 2-way ANOVA followed by Sidak's multiple comparisons, $*p < 0.05$. CA - closed arms, OA - open arms.

Figure S2

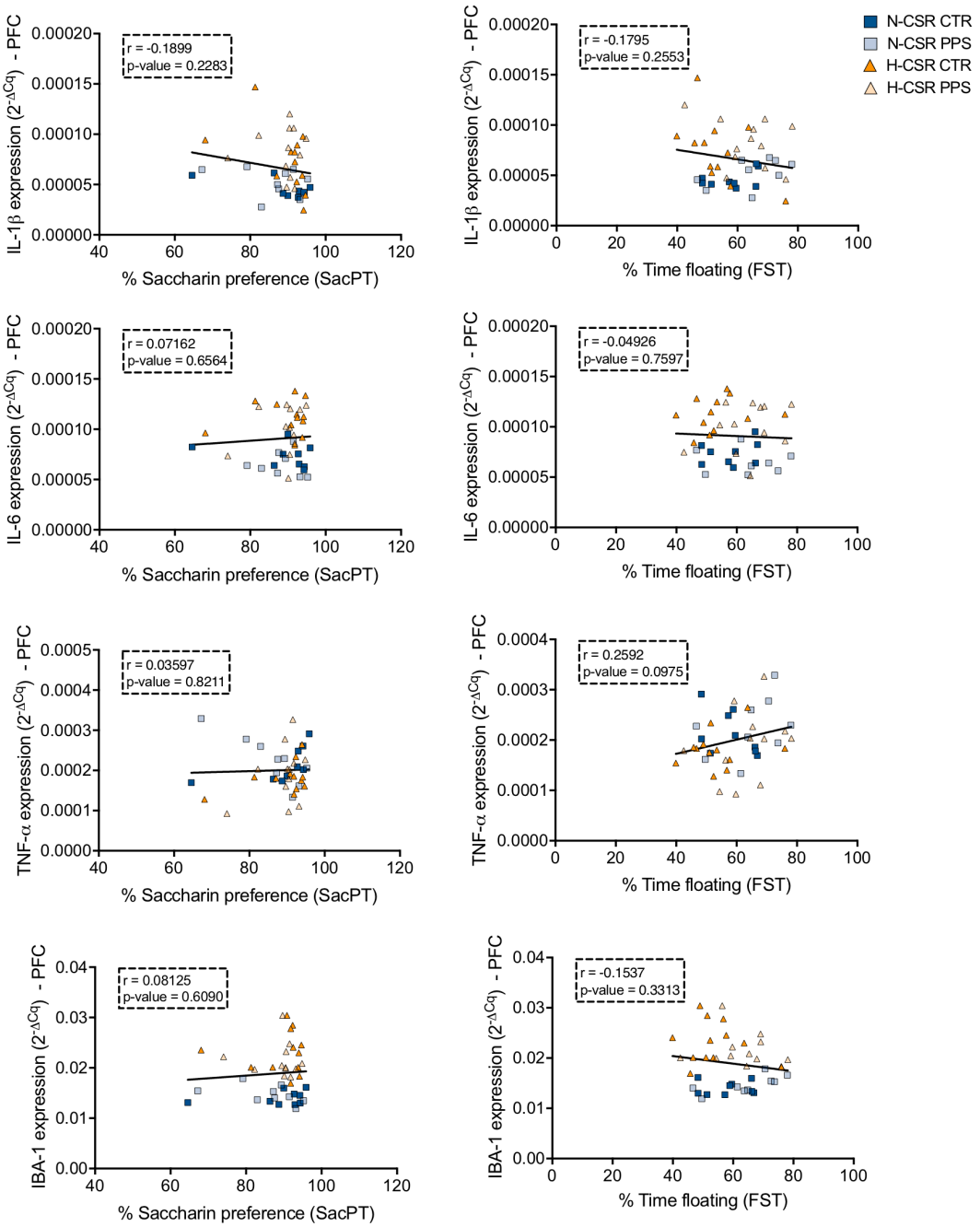


Figure S2. Correlations between the expression levels of inflammatory markers in the mPFC and depressive-like behaviours. Pearson correlations were performed by comparing mRNA expression levels of IL-1β, IL-6, TNF-α and IBA-1 in the medial pre-frontal cortex (mPFC) and % saccharin preference (SacPT) or % time floating (FST). Correlations were performed considering all animals (N=42). Coefficient r and *p*-value for each correlation are presented in a box. Statistically significant correlations are highlighted in green (p<0.05).

Figure S3

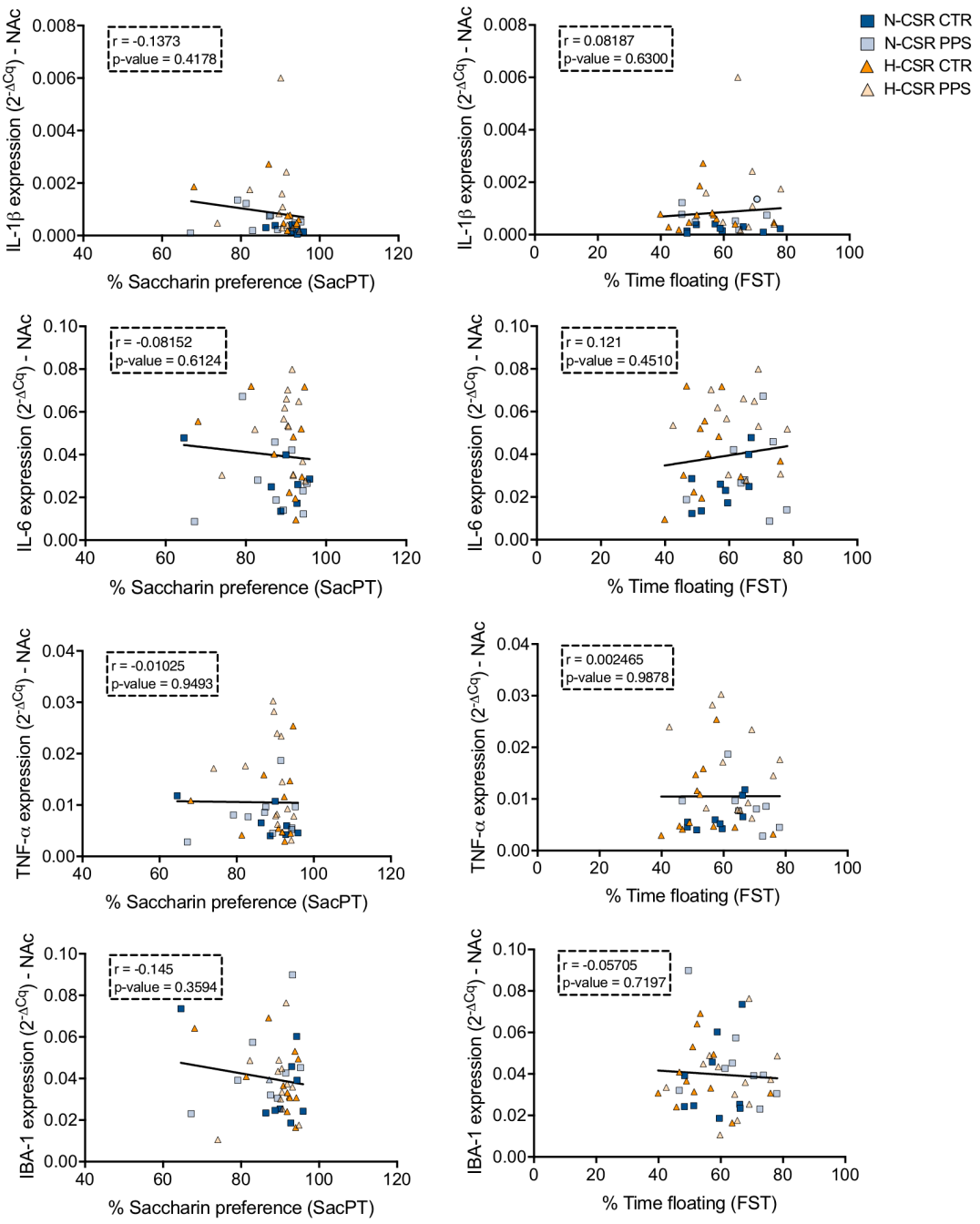


Figure S3. Correlations between the expression levels of inflammatory markers in the NAc and depressive-like behaviours. Pearson correlations were performed by comparing mRNA expression levels of IL-1β, IL-6, TNF-α and IBA-1 in the nucleus accumbens (NAc) and % saccharin preference (SacPT) or % time floating (FST). Correlations were performed considering all animals (N=42). Coefficient r and *p*-value for each correlation are presented in a box. Statistically significant correlations are highlighted in green (p<0.05).

Figure S4

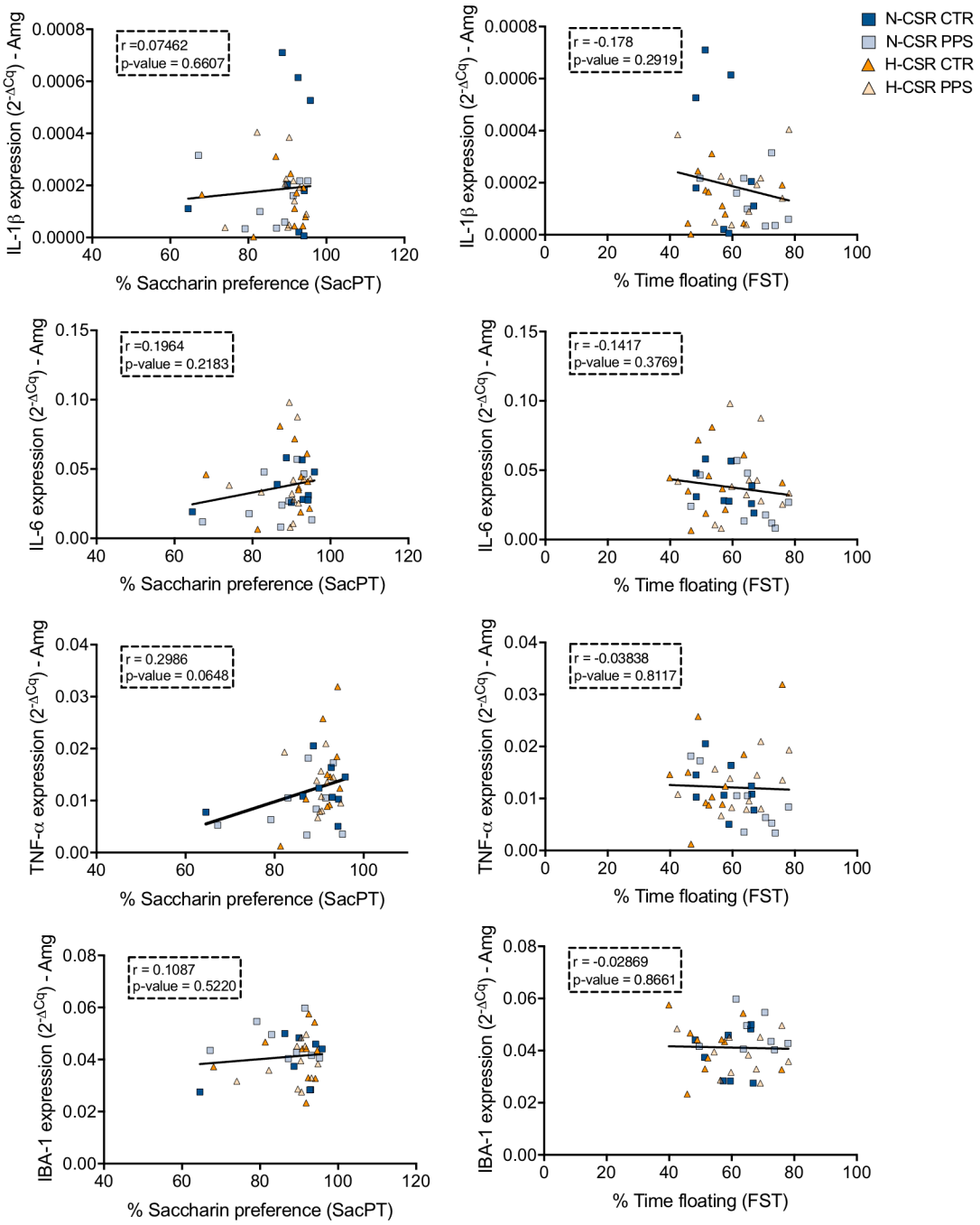


Figure S4. Correlations between the expression levels of inflammatory markers in the Amg and depressive-like behaviours. Pearson correlations were performed by comparing mRNA expression levels of IL-1β, IL-6, TNF-α and IBA-1 in the amygdala (Amg) and % saccharin preference (SacPT) or % time floating (FST). Correlations were performed considering all animals (N=42). Coefficient r and *p*-value for each correlation are presented in a box. Statistically significant correlations are highlighted in green ($p<0.05$).

Figure S5

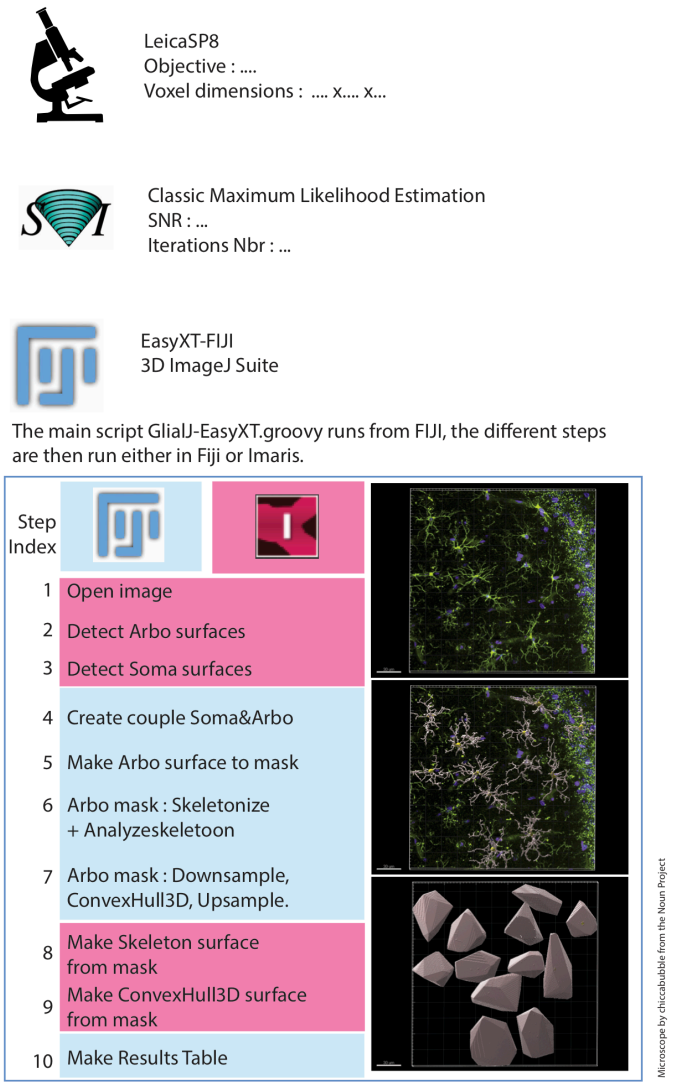


Figure S5. Microglia 3D morphological analysis: step-by-step. Images were acquired using Leica SP8 with a 40x glycerin immersion objective. The pixel size was set to 0.230 microns. The acquired images were deconvolved using SVI Huygens Professional called via Huygens Remote Manager v3.7, using the "Classic Maximum Likelihood Estimation" algorithm. The analysis of the deconvolved images was performed with Fiji and Imaris using the EasyXT-Fiji plugin via a custom groovy script. Imaris surfaces were created to segment the arborization of the cells and for their soma. Next, cells were created by linking the soma to its corresponding arborization, based on the nearest centers of mass (Step Index 1-3). Next, for each cell, the mask of the arborization was sent to Fiji: 1) skeletonized and analyzed (average and maximum branch length, number of branches), and 2) down sampled, in order to calculate its 3D ConvexHull, which was then upscaled back to the original size (Step Index 4-7). Finally, the masks of the Skeleton and of the 3D ConvexHull were sent back to the Imaris Scene (Step Index 8 and 9) and a results file was created with volumes and calculated ratios (Step Index 10).

Figure S6

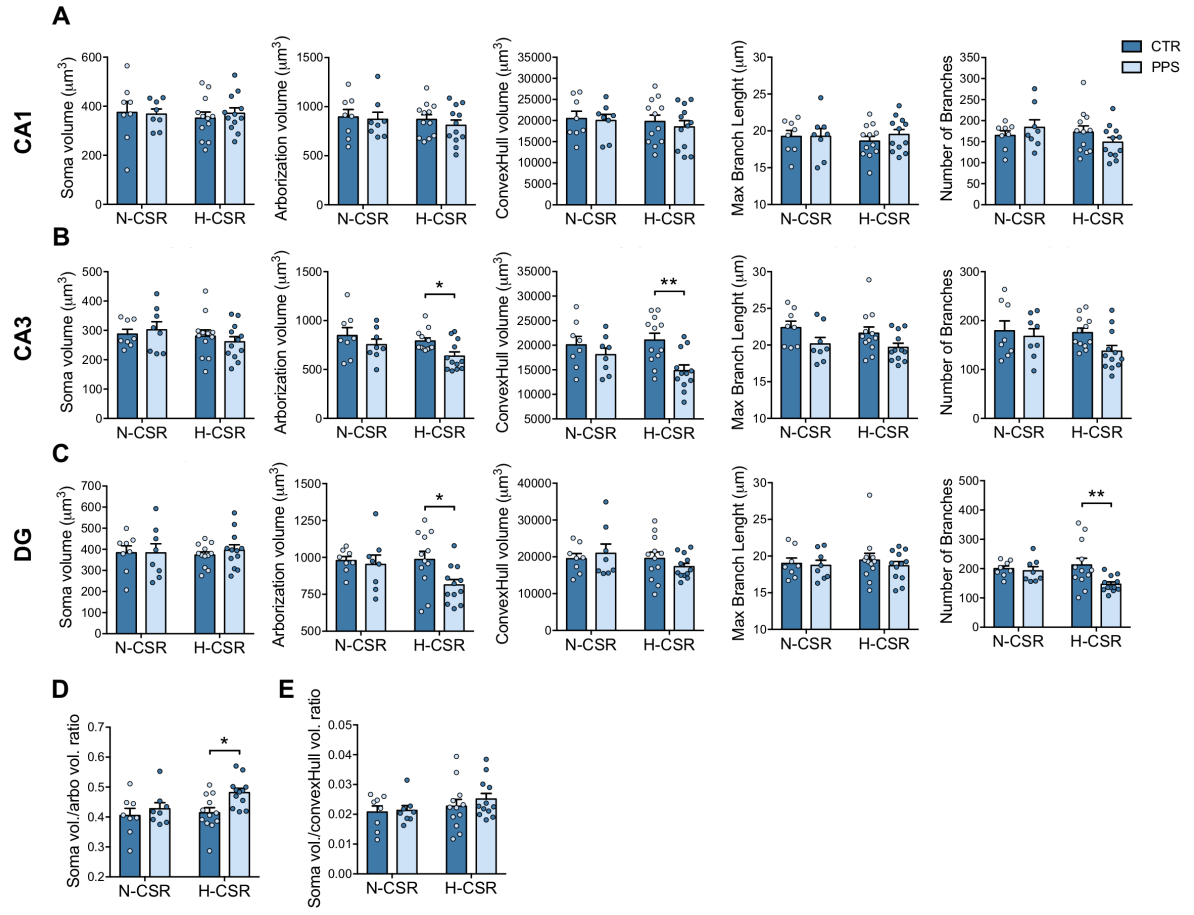


Figure S6. Quantitative analysis of microglial morphology in CA1, CA3 and DG hippocampal subregions. Results represent microglial morphological parameters quantified in the CA1 (A), CA3 (B) and DG (C) subregions. On average, more than 20 cells per animal were analysed in terms of soma volume, arborization volume, convexHull volume, max branch length and number of branches. Graph (D) show the ratio between soma volume and arborization volume and (E) the ratio between soma volume and ConvexHull volume, considering the average values of the 3 fields (CA1, CA3 and DG). Each dot represents an animal (N: N-CSR = 18 (9 CTR and 9 PPS); H-CSR = 24 (12 CTR and 12 PPS). Results are expressed as mean ± SEM. 2-way ANOVA followed by Sidak's multiple comparisons, *p < 0.05.

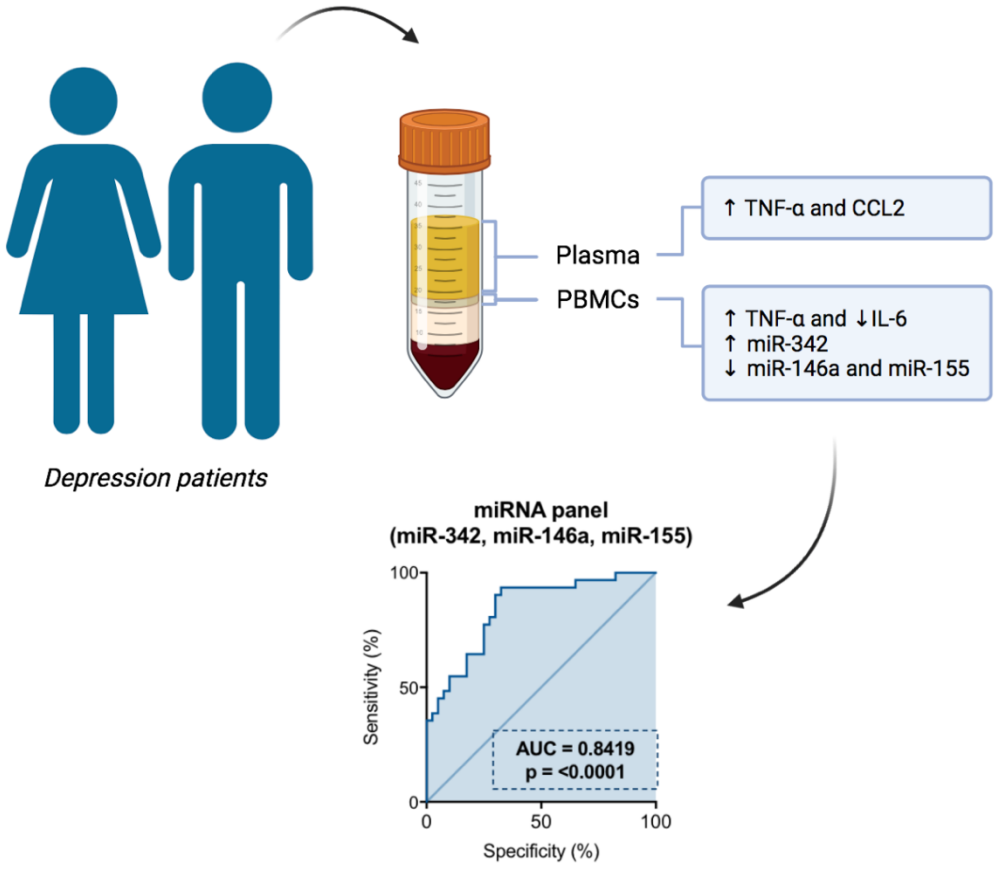
3. A clinical study of inflammatory and miRNA mediators in depression

The content of this chapter was compiled in a manuscript that is in preparation for submission.

Article IV

João P. Brás, Sara Pinto, Joana Prata, Orlando von Doellinger, Rui Coelho, Mário A. Barbosa, Maria Inês Almeida, Susana G. Santos. **A clinical study of inflammatory and miRNA mediators in depression.** *In preparation*

Graphical abstract



Graphical abstract. Clinical study of inflammatory and miRNA mediators in depression. Here we show that depression patients have increased systemic inflammation, reflected on increased plasma levels of TNF-α and CCL2, increased TNF-α mRNA levels in PBMCs, and dysregulated expression of key inflammation-related miRNAs in PBMCs. ROC analysis revealed that, when used in combination to distinguish between depression patients and healthy controls, miR-342, miR-146a and miR-155, have potential to constitute a good diagnostic panel, that should be further investigated in the future.

Abstract

In recent years, inflammation has been implicated in core features of depression pathophysiology and treatment resistance. Therefore, new challenges in the discovery of inflammatory mediators implicated in depression have emerged. microRNAs (miRNAs) have been found aberrantly expressed in several pathologies, increasing their potential as biomarkers and therapeutical targets. In this study, the aim was to assess the changes and biomarker potential of inflammation-related miRNAs in depression patients. Depression diagnosis was performed according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). 40 healthy controls and 32 depression patients prior treatment were included in the study. The levels of inflammatory cytokines were measured in plasma, and expression levels of cytokines and miR-145, miR-146a, miR-155 and miR-342 were evaluated in peripheral blood mononuclear cells (PBMCs). Depression patients were found to have a pro-inflammatory profile in plasma, with significantly higher levels of TNF-α and CCL2 compared with healthy controls. In PBMCs of depression patients, TNF-α and IL-6 expression levels were significantly up and downregulated, respectively. Moreover, miR-342 levels were found upregulated, while miR-146a and miR-155 were significantly downregulated in PBMCs of depression patients. Of note, PBMCs' expression levels of miR-342 were positively correlated with TNF-α. Importantly, when analysed as diagnostic panel, ROC analysis of miR-342, miR-146a, miR-155 in combination, showed to be highly significant, with an area under the curve of 0.8419 (CI=0.75-0.93, $p<0.0001$). In summary, these findings suggest that inflammation-related miRNAs are aberrantly expressed in depression patients, opening new possibilities for the use of miRNA panels as depression biological biomarkers.

Keywords: Depression, inflammation, microRNAs, cytokines, PBMCs

3.1. Introduction

Depression ranks as the most prevalent psychiatric disorder, with estimated over 260 million sufferers worldwide (WHO2020), and a lifetime prevalence of 10-20% (1). It also figures among the top three causes of disability worldwide and can affect individuals of all ages throughout their entire lifespan, with a higher prevalence in women (2). Conventional pharmacological treatments for depression are mainly based on the monoamines theory of depression, targeting neurotransmission regulation (3). However, it is estimated that between 30 to 50% of patients with major depression do not respond to the prescribed schemes of antidepressant medication (4-6), reinforcing the need to stratify patients, and understand the multifactorial aetiology of this disorder. It is now clear that the major reason, still preventing a most accurate diagnosis as well as the development of better pharmacotherapies, is the poor understanding of the molecular pathology underlying depression. This leads to narrative and observation-based diagnosis, disregarding the biological particularities of each patient (7). Thus, there is an urgent need to establish complementary diagnostics tests, by defining diagnosis and prognosis biomarkers, as well as to develop a wider spectrum of novel therapeutics to target other possible underlying disease mechanisms.

Over the last decades, research has strongly focused on the inflammatory/immune hypothesis of depression, with most treatment resistant patients presenting a hyper-activation of the immune system (8-10). Clinical presentation of depression has long been compared with the so called “sickness behaviour”, which occurs when individuals suffer from an inflammatory/infectious disease (11, 12). Moreover, the relation between depression and inflammation has been strongly suggested by patients with chronic inflammatory conditions, such as rheumatoid arthritis, psoriasis, inflammatory bowel disease and multiple sclerosis, among others (13-17). While the incidence of depression in patients with chronic inflammatory diseases is substantially higher, acute exacerbations of these diseases may also be preceded by stressful events or depressive episodes (9). Based on these findings, clinical trials have been exploring the potential of anti-inflammatory therapies to treat depression. While most of the reports suggest that anti-inflammatory agents play an efficient antidepressant role and are reasonably safe (18), others cast doubts on the potential therapeutic benefits of adjunctive anti-inflammatory drugs for the acute management of depression (19). Nonetheless, it is evident that a move away from symptom-based to a biological-based diagnosis is urgently needed. Innovative trial designs, with biologically based clinical outcomes, and more selective drugs, will prevent researchers from failing to take advantage of the increasing knowledge regarding the role of inflammation in depression.

In this sense, microRNAs (miRNAs) have recently emerged as important mediators in the pathophysiology of inflammation-related depression, with potential to be used as therapeutical

targets and/or biomarkers (20-22). miRNAs are small non-coding RNAs that regulate multiple target transcripts, influencing entire gene networks in processes such as inflammation (inflammiRs), neurogenesis and neuronal plasticity (23-25). Recently, our group found that miR-342 is upregulated in TNF-α activated microglia, and its overexpression leads to increased secretion of inflammatory mediators, drastically affecting neuron viability (26). miR-155 is known to have a dual role depending on the inflammatory stage, by acutely function as a strong promotor of anti-pathogen responses and lately limit the strength of the resulting NF-κB dependent inflammatory response (27, 28). On the other hand, miR-145 and miR-146a have been recurrently associated with anti-inflammatory, neurogenesis and neuroprotective mechanisms (29-32). Based on these features, we hypothesize that these inflammiRs may appear dysregulated in depression, with strong genetic support for associating them and their targets with this condition.

In this study, we evaluated the levels of inflammatory cytokines in plasma and PBMCs, and the expression levels of miR-145, miR-146a, miR-155 and miR-342 in PBMCs. The results show a pro-inflammatory profile in plasma of depression patients, with increased TNF-α and CCL2, as well as significant correlations between the levels of different pro- and anti-inflammatory mediators. In PBMCs, TNF-α and IL-6 expression levels were significantly up and downregulated, respectively. Also, miRNA-342 was found significantly upregulated, while miRNA-155 and miRNA-146a were significantly downregulated in depression patients compared with healthy controls. The levels of these miRNAs corelated with those of the inflammatory cytokines, and ROC analysis of their levels showed high significance and area under the curve, when miRNA-342, miRNA-155 and miRNA-146a were considered together. In summary, the results presented here open new possibilities for the use of miRNA panels as depression biological biomarkers.

3.2. Materials and methods

3.2.1. Ethics statement

All obtained human samples and procedures were performed in agreement with the principles of the Declaration of Helsinki. Blood samples were collected from patients enrolled at the psychiatry departments of *Centro Hospitalar do Tâmega e Sousa, EPE* and *Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE*; and from healthy blood donors, at *Serviço de Imunohemoterapia*, *Centro Hospitalar Universitário de São João*, after informed consent. All experimental protocols were conducted following the approval and recommendations of the Ethics Committees for the hospitals involved.

3.2.2. Experimental design

Patients admitted to the Department of Psychiatry and Mental Health of *Centro Hospitalar do Tâmega e Sousa* (Penafiel, Portugal), and outpatients from the Department of Psychiatry and Mental Health of *Centro Hospitalar Vila Nova de Gaia/Espinho* (Vila Nova de Gaia, Portugal), diagnosed with major depression and that agreed to take part, were enrolled in the study. Participants ranged in age from 18–65 years and entered the study after screening and diagnosis of major depressive disorder was confirmed by two psychiatrists, as described previously [18,39]. Briefly, screening included a structured clinical interview to assess the presence of major psychiatric syndromes according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), an assessment of current psychiatric symptoms, and a determination of previous antidepressant treatment. Blood samples were obtained from depression patients at baseline, before undergoing treatment. Single blood samples were also obtained from healthy control subjects at the *Serviço de Imunohemoterapia* do *Centro Hospitalar Universitário de São João* (Porto, Portugal). Healthy control subjects were screened to rule out a personal or family history (first-degree relative) of psychiatric disorder. Patients and controls were excluded from the study if presenting any of the following: i) psychotic symptoms; ii) presence of an infectious or inflammatory illness or the regular use of anti-inflammatory medication; iii) Inability to completely understand and fill in the self-assessment instruments; iv) Being part of another study or with other psychological/psychopharmacological treatment.

3.2.3. Plasma and PBMCs isolation

Peripheral blood was collected using VACUETTE® Tubes EDTA K3 (Greiner Bio-One, France). Blood components were separated by centrifuging at 1200g, for 20 min, at RT, without break. Plasma was collected and centrifuged twice at 2500g, for 10 min at 4ºC before being aliquoted and stored at -80ºC. Medial layer containing PBMCs was slowly collected and transferred into a new 15mL centrifuge tube. PBMCs were diluted in an equal volume of PBS 1x, slowly layered over Lymphoprep (Ratio 1:1) and centrifuged at 800g, for 20min, at RT, without break. Medial layer containing enriched PBMCs was collected and cells washed twice with PBS 1x (300g, 10min, at 4ºC) before being lysed with TRIzol®.

3.2.4. RNA extraction

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were evaluated in a NanoDrop 1000 (Thermo Scientific). Ratios of 260/280 and 260/230 nm ranged between 1.8 and 2.2.

RNA integrity was evaluated by agarose gel electrophoresis or by Experion™ automated electrophoresis system (Bio-Rad, USA).

3.2.5. Reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR)

For gene expression analysis, RNA was treated with TURBO DNA-free Kit (Invitrogen) and cDNA was synthesized using Random Hexamers (Invitrogen), dNTPs (Bioline) and SuperScript® III Reverse Transcriptase (Invitrogen). qPCR was carried out in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) using cDNA, primers and iQ SYBR Green Supermix (Bio-Rad). GAPDH was used as reference gene. Oligonucleotides used for qPCR experiments are shown in (Supplementary Table 1).

miR-145-5p, miR-146a-5p, miR-155-5p and miR-342-3p expression was evaluated using TaqMan miRNA assays (Applied Biosystems). Briefly, cDNA was synthesized using 30 ng of RNA as a template, gene-specific stem-loop Reverse Transcription primer, and the TaqMan microRNA reverse transcription kit (Applied Biosystems). qPCR was carried out in CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) using cDNA, TaqMan probe and SsoAdvancedTM Universal Probes Supermix (Bio-Rad). Small nuclear RNA U6 was used as reference gene. All runs were performed in duplicate. Relative expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines (33).

3.2.6. Enzyme-linked immunosorbent assay (ELISA)

CCL2, IL-6, IL-1β, TNF-α, IL-4 and IL-10 levels were evaluated by ELISA, according to the manufacturer's instructions (ELISA MAX™ Deluxe Set, BioLegend, USA). Absorbance was measured in a plate reader at 450 nm, with wavelength correction at 570 nm. Cytokine concentrations (pg/mL) were determined using a standard calibration curve.

3.2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, Inc.). Gaussian distribution was tested by the D'Agostino & Pearson and the Shapiro-Wilk normality tests. For non-normal distribution data, statistical differences were evaluated by unpaired Mann-Whitney rank test. When data passed normality test, unpaired *t*-tests were performed. Spearman correlation analyses (non-parametric data) between plasma cytokines, cytokine mRNA levels and miRNA levels in PBMCs were performed considering only depression subjects. The total number of individuals and statistical tests used are identified in each figure legend. Statistical significance was considered for $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: non-significant). The diagnostic value of the tested miRNAs (individually or combined) was calculated using Receiver Operating Characteristics (ROC)

curve in GraphPad. For that, miRNAs relative expression values were previously combined using the binary logistic toll on SPSS considering the experimental group as the dependent variable and the different tested miRNAs as covariates. The area under the curve (AUC) measures of the ability of each miRNA or the miRNA panel to distinguish between both groups, and the *p*-value tests the null hypothesis that the AUC equals 0.5.

3.3. Results

3.3.1. Depression patients exhibit increased levels of inflammatory mediators in plasma

A total of 72 subjects were included in the study. The depression group was composed of 32 subjects (27 females and 5 males) with a mean age of 38.81 ± 2.13, while 40 healthy subjects (23 females and 17 males), with a mean age of 37.95 ± 1.71, were included in the control group (Table 1).

Table 1. Demographic characteristics of depression patients and healthy controls.

	Depression	Controls
N	32	40
Sex (F/M)	27/5	23/17
Age ± SEM	38.81 ± 2.13	37.95 ± 1.71

Dysregulated levels of inflammatory mediators have been described in depression patients (8), so the plasma levels of several inflammatory markers were evaluated by ELISA. Results revealed that the classical pro-inflammatory cytokine TNF-α (p=0.0011) and also the CCL2 chemokine (p=0.0453), were significantly upregulated in depression patients compared with healthy controls (Figure 1). Although not significantly altered, IL-6 levels tend to be upregulated (p=0.0691) and IL-4 downregulated (p=0.0746) in depression patients (Figure 1). Plasma levels of IL-1β and IL-10 were not significantly different between depression patients and healthy controls (Figure 1). Next, Spearman correlations were used to understand the correlation between the levels of these different inflammatory mediators. The results revealed that depression patients' plasma levels of CCL2, IL-6, IL-4 and IL-10 were all positively correlated (Supplementary Table 2).

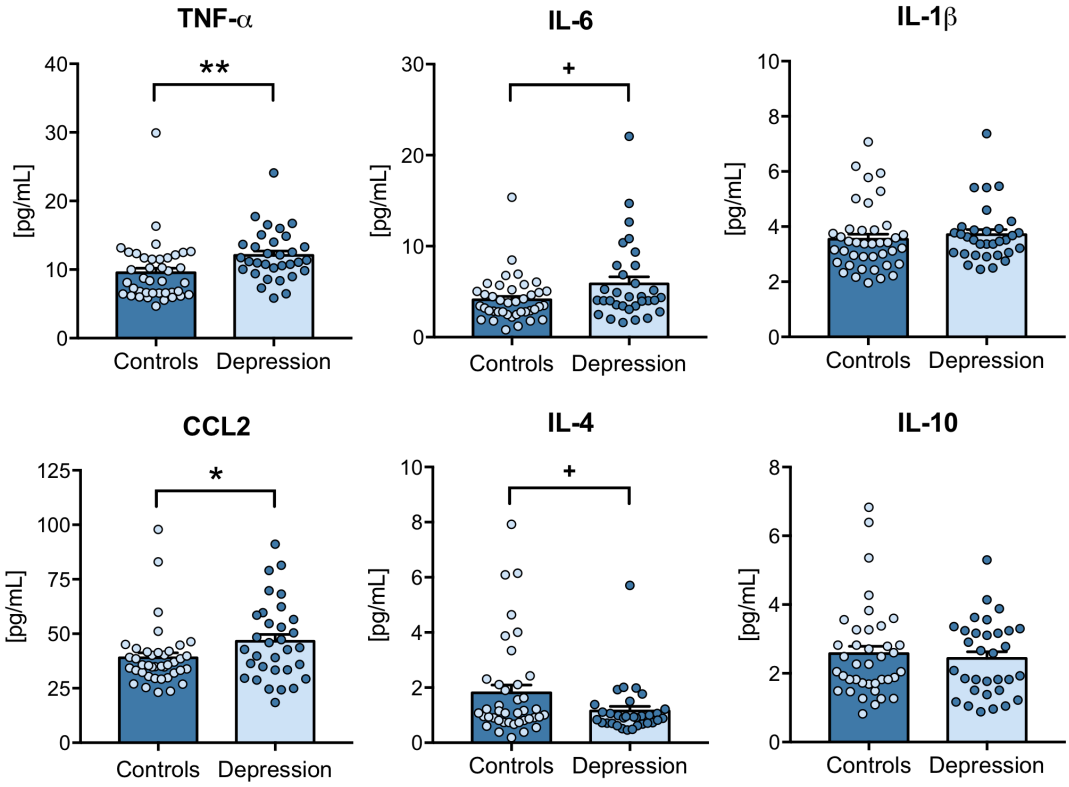


Figure 1. Plasma levels of inflammatory cytokines in depression patients versus healthy controls. Blood components were separated, and plasma was used to measure CCL2, IL-6, IL-1β, TNF-α, IL-4 and IL-10 levels by ELISA, according to the manufacturer's instructions. All samples were tested simultaneously and under the same conditions for each cytokine. Cytokine concentrations ([pg/mL]) were determined using a standard calibration curve, and results are presented as mean ± SEM. Each dot represents an individual (control or depression patient). Statistical differences between groups were evaluated using Mann-Whitney non-parametric unpaired test (*** p<0.001, + p<0.1). N: Healthy controls = 40; Depression patients = 32.

3.3.2. TNF-α expression is increased in PBMCs of depression patients

To further explore the contribution of circulating immune cells to the changes observed on inflammatory markers, their mRNA levels were evaluated in PBMCs by RT-qPCR. Results revealed that, in agreement with the increased plasma levels, TNF-α mRNA levels were significantly upregulated (p=0.0073). Interestingly, while protein levels of IL-6 in plasma showed a tendency for increase, its mRNA levels in PBMC were significantly downregulated (p<0.001) in depression patients compared with healthy controls (Figure 2). Although IL-1β mRNA levels tend be upregulated in depression patients (p= 0.0606, Figure 2), no significant differences were found between the mRNA levels of IL-1β nor CCL2 in PBMCs of depression patients and healthy controls. Interestingly, a positive Spearman correlation was maintained at the mRNA level in PBMCs of depression patients, between CCL2, IL-6 and TNF-α (Supplementary Table 3).

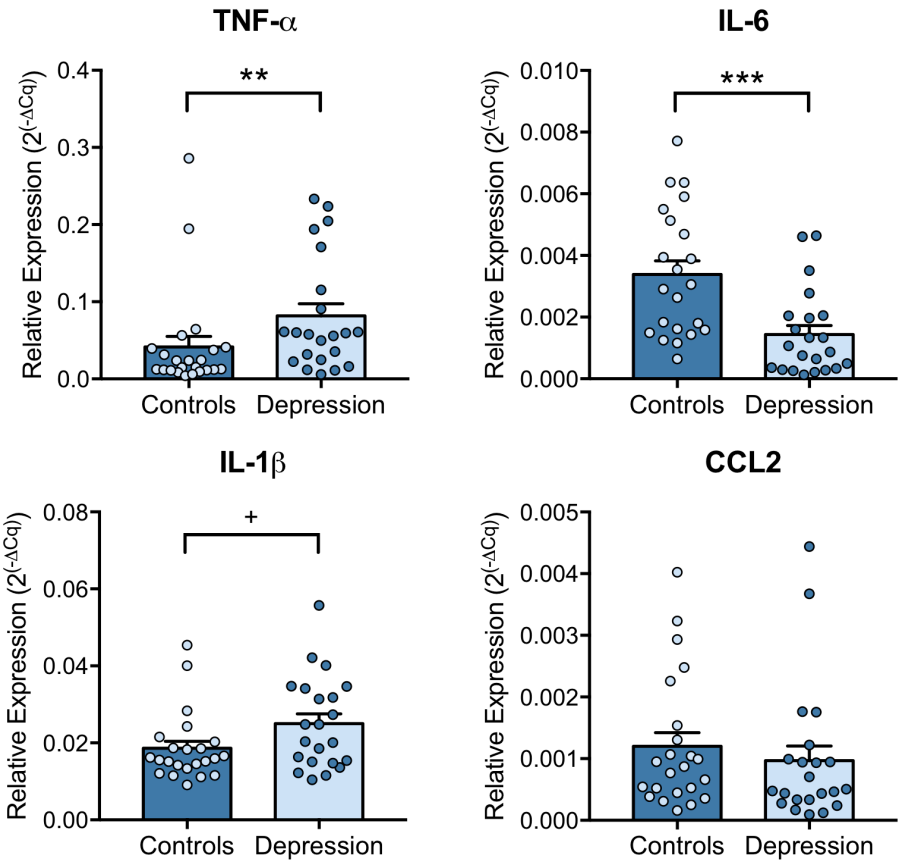


Figure 2. Levels of pro-inflammatory cytokines in PBMCs of depression patients versus healthy controls. Blood components were separated, PBMCs collected, and RNA extracted using TRIzol. CCL2, IL-6, IL-1β, TNF-α mRNA levels in PBMCs were evaluated by RT-qPCR using GAPDH as internal control. Relative expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines, and results are presented as mean ± SEM. Each dot represents an individual (Control or Depression patient). Statistical differences between groups were evaluated using Mann-Whitney non-parametric unpaired test (*** $p<0.001$, ** $p<0.01$ and + $p<0.1$). N: Healthy controls = 23; Depression patients = 22.

3.3.3. Inflammatory miRNAs are aberrantly expressed in PBMCs of depression patients

Next, the regulation of inflammatory markers was explored, by investigating the levels of several miRNAs that can target inflammatory mediators, like TNF-α. miR-342, miR-145, miR-146a and miR-155 expression levels in PBMCs of depression patients and healthy controls were evaluated by RT-qPCR. Results revealed that miR-342 is significantly upregulated ($p=0.0117$), while miR-146a ($p<0.001$) and miR-155 ($p=0.0056$) levels are significantly downregulated in PBMCs of depression patients compared with healthy controls (Figure 3). miR-145 expression levels tend to be downregulated in PBMCs of depression patients, but this decrease is not statistically significant ($p=0.067$, Figure 3). No statistically significant

correlations were observed between the expression levels of the tested miRNAs in PBMCs of depression patients (Supplementary Table 4). However, Spearman correlation analysis showed that TNF-α mRNA levels were significantly positively correlated with miR-342 (r=0.4274, p=0.0472), while IL-6 mRNA levels were significantly correlated with miR-145 levels (r=0.4850, p=0.0221). On the other hand, IL-1β mRNA levels were negatively correlated with miR-155 levels (-0.5697, p=0.0056, Table 2).

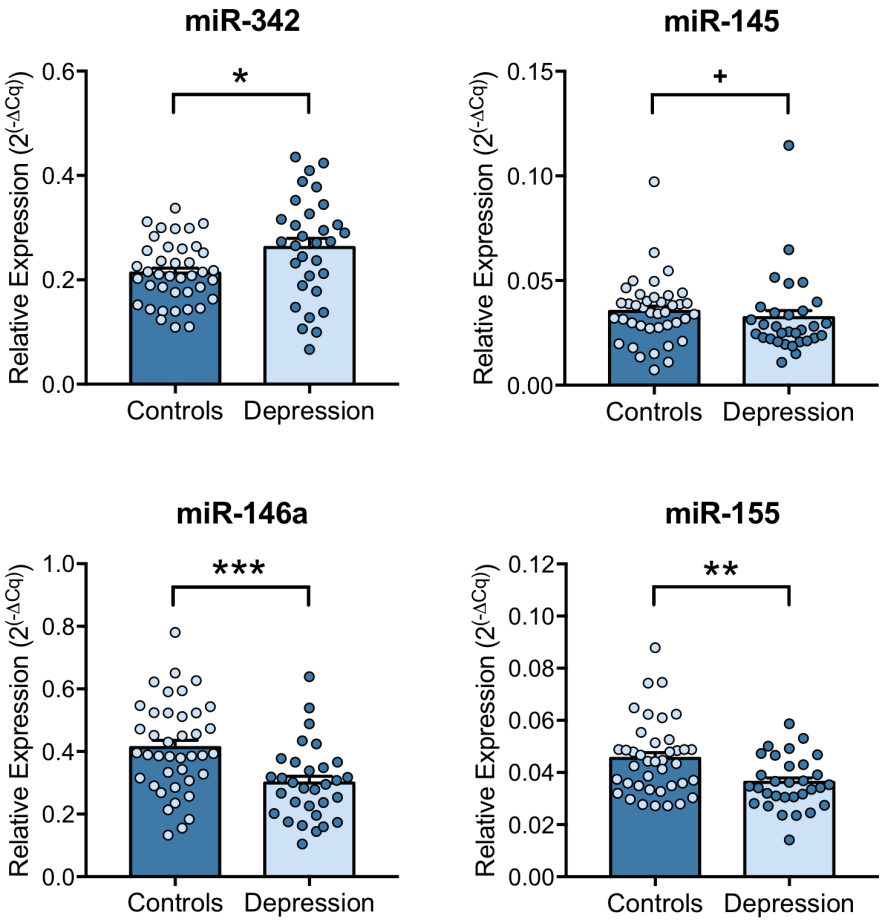


Figure 3. miRNA expression levels in PBMCs of depression patients versus healthy controls. miR-342, miR-145, miR-146a and miR-155 expression levels in PBMCs were evaluated by RT-qPCR using U6 snRNA as internal control. Relative expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines, and results are presented as mean ± SEM. Each dot represents an individual (Control or Depression patient). Statistical differences between groups were evaluated using Mann-Whitney non-parametric unpaired test or unpaired *t*-test (** p<0.01, * p<0.05 and + p<0.1). N: Healthy controls = 40; Depression patients = 32.

Table 2. Spearman correlations between cytokines mRNA levels and miRNA levels in PBMCs of depression patients. The coefficient r and *p*-value for each correlation are presented. Statistically significant correlations are highlighted (p<0.05).

		PBMCs							
		miR-342		**miR-145**		**miR-146a**		**miR-155**	
		r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
PBMCs	**CCL2**	0.3145	*0.1539*	0.4150	*0.0547*	0.2275	*0.3084*	0.2320	*0.2986*
	IL-6	0.3642	*0.0956*	**0.4850**	***0.0221***	-0.0254	*0.9106*	0.0592	*0.7932*
	IL-1β	-0.2795	*0.2077*	0.0367	*0.8711*	-0.1383	*0.5392*	**-0.5697**	***0.0056***
	TNF-α	**0.4274**	***0.0472***	0.3879	*0.0744*	-0.0909	*0.6874*	0.1507	*0.5030*

Finally, the potential for these miRNAs to be used as biological quantitative diagnostic markers for depression was explored. ROC analysis was performed to evaluate the ability of the differently expressed miRNAs to distinguish between depression patients and healthy controls, individually or in combination. The area under the curve (AUC) for miR-342, miR-146a and miR-155 when tested individually was 0.667 (CI=0.53-0.80, p=0.0164), 0.736 (CI=0.62-0.85, p=0.0007) and 0.691 (CI=0.57-0.81, p=0.006) respectively. When tested as a miRNA panel, sensitivity increased significantly and the use of the three miRNAs in combination was the best classifier (AUC=0.842, CI=0.75-0.93, p<0.0001; Figure 4 and Supplementary Table 5).

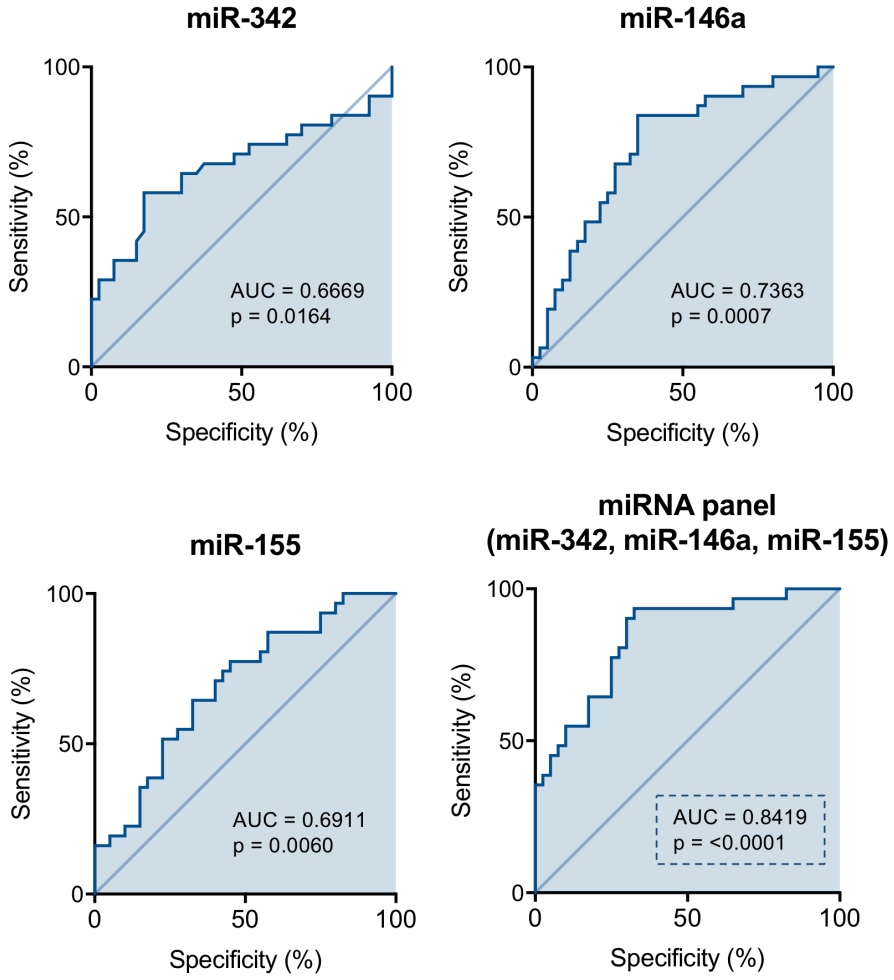


Figure 4. ROC analysis of the differently expressed miRNAs. ROC analysis was performed to evaluate the sensitivity of miR-342, miR-146a and miR-155 to distinguish between depression patients and controls when used individually or in combination as a miRNA panel. The area under the curve (AUC) represents the measure of the ability of each miRNA or the miRNA panel to distinguish between both groups, and the *p*-value tests the null hypothesis AUC equals 0.5. N: Healthy controls = 40; Depression patients = 32.

3.4. Discussion

In this study, we evaluated whether the levels of inflammatory cytokines and the expression levels of miR-145, miR-146a, miR-155 and miR-342 in PBMCs, were altered in depression patients prior antidepressant treatment, compared with healthy controls. Depression patients were found to have significantly higher levels of TNF-α and CCL2 in the plasma, and increased expression levels of TNF-α together with decreased IL-6, in PBMCs. Moreover, miR-342 levels were found upregulated, while miR-146a and miR-155 were significantly downregulated in PBMCs of depression patients. Of note, expression levels of miR-342 positively correlated with those of TNF-α in PBMCs. Importantly, when these three miRNAs were analysed as

diagnostic panel, their ROC analysis, showed to be highly significant, with an area under the curve of 0.8419.

In recent years, the immune system has emerged as a key player in depression symptomatology and treatment resistance (34). A segment of depression patients exhibits chronic inflammation, shown by increased systemic levels of inflammation-related markers (8). In this study, we found an upregulation of TNF-α and CCL2 levels in the plasma of depression patients, before treatment started. These findings are in accordance with the literature, as TNF-α and CCL2 have been recurrently found upregulated in depression patients prior treatment and in treatment-resistant patients (8, 35). On the other hand, plasma levels of IL-6 and IL-1β were not significantly upregulated in the segment of patients analysed. Despite being frequently dysregulated in inflammation-related disorders, IL-1β has been found unaltered in depression (36). In the case of IL-6, literature has been supporting an increase in plasma of depression patients (37). Although there is a tendency to be upregulated, no significant differences were found for IL-6 in depression patients *versus* healthy controls. Regarding anti-inflammatory cytokines IL-4 and IL-10 were unchanged between depression patients and controls. A recent report by Yuan *et al.*, assessing reproducibility and specificity of inflammation-related markers in major psychiatric disorders, also found no changes in plasma levels of IL-4 and reported inconsistent findings on the levels of IL-10 in depression (36).

Inflammation is known to play a role in core features of depression, particularly due to the action of central nervous system (CNS) activated microglia and astrocytes, infiltrated and peripheral BMCs (particularly monocytes/macrophages and T lymphocytes), as well as the immunoreactive molecules (e.g., cytokines and chemokines) they release (38, 39). Since brain tissue is rarely available for study, the analysis of other peripheral information sources, such as saliva, plasma, serum and particularly PBMCs, has received increasing attention. In fact, previous studies have shown that peripheral blood cells share more than 80% of the transcriptome with brain tissue, therefore offering a potential diagnostic tool that can dynamically reflect changes in brain macro- and micro-environments (40). In this sense, we analysed the expression levels of several inflammatory molecules in PBMCs of both depression and healthy subjects. In line with what was observed in plasma, TNF-α mRNA levels were found upregulated in depression patients compared with healthy controls, suggesting that PBMCs might be contributing to the observed increased levels in plasma. On the contrary, while CCL2 levels in plasma were found upregulated in depression patients, mRNA levels of this cytokine in PBMCs remained unaltered. Moreover, despite a tendency to an increase in plasma, IL-6 mRNA levels were found downregulated in the PBMCs of depression patients, in what we hypothesize could be a mechanism of negative feedback, to

compensate the increased levels of IL-6 and other dysregulated cytokines in plasma. In fact, this disconnection between cytokine plasma levels and circulating inflammatory cells phenotype was also reported before (41). Hasselmann *et al.* found that despite depression patients showing higher frequency and higher absolute numbers of non-classical monocytes, there was no correlation between those changes and circulating levels of CRP, IL-6, IL-1β, or TNF-α (41). Although being frequently overlapped, these findings support the need to distinguish between cytokine levels measured in plasma/serum and mRNA levels detected in circulating inflammatory cells, in order to fully understand the contribution of immune cells and the cascade of activation states they go through during chronic systemic inflammation.

miRNAs' unique expression patterns and ability to modulate mRNA levels of a large number of target genes, often related to disease-associated pathological processes, such as in inflammation-related depression, increases their desirability as diagnostic markers (42-44). In this study, an overexpression of miR-342 and a downregulation of miR-146a and miR-155 in the PBMCs of depression patients were found. Previously, using mouse *in vitro* cultures, our group identified miR-342 as a crucial mediator of TNF-α-driven microglia activation. After being found upregulated in TNF-α stimulated microglia, miR-342 microglial overexpression *per se* was shown to be sufficient to induce neurotoxicity, activating the NF-κB pathway, and leading to increased microglial secretion of TNF-α and IL-1β (26). Microglia activation has been associated with neurodegenerative diseases and psychiatric disorders, including depression (45). In response to adverse stimuli, such as psychological stress, brain injuries or infections, microglia overproduce proinflammatory cytokines and chemokines that not only influence the surrounding microenvironment but also promote the recruitment of peripheral immune cells (46, 47). This results in exacerbated neuroinflammation, leading to an imbalance of several brain functions, some of which characteristic of depression (45, 48). To our knowledge, this is the first study reporting increased expression levels of miR-342 in depression patients. Importantly, miR-342 levels were positively correlated with TNF-α levels, showing that a strong interplay between TNF-α and miR-342 is also found in humans, and outside the brain in circulating cells. miR-146a has been shown to attenuate deleterious processes associated with dysregulated inflammation in several diseases, including rheumatoid arthritis and atopic dermatitis (49). miR-146a acts as a mitigator of inflammatory responses by targeting key molecules of NF-κB and JAK-STAT signalling pathways, thereby reducing pro-inflammatory cytokines production, such as TNF-α and IL-8 (50, 51). In pathologies with chronic low-grade baseline inflammation, miR-146a have been recurrently associated with miR-146a downregulation (52-54), indicating that its dysregulation contributes to pathology. Previously, miR-146a was found downregulated in the prefrontal cortex of suicide victims diagnosed with clinical depression (55). Here, we show that miR-146a levels are also downregulated in the PBMCs of depression patients, which may partially explain the

observed increase of TNF-α levels. In agreement, a recent study performed by Hung *et al.* evaluating the expression levels of intracellular miRNAs that regulate TLR4 signaling in PBMCs and monocytes of depression patients, found a downregulation of miR-146a and miR-155 in PBMCs (56). In the current study, we also found miR-155 levels downregulated in PBMCs of depression patients. miR-155 is known as a master regulator of inflammation performing both pro- and anti-inflammatory functions (57). miR-155 is normally found upregulated in acute inflammatory responses as its expression is highly induced by TLR ligands/activation (58). In early inflammatory responses stages, miR-155 targets the suppressor of cytokine signaling 1 (SOCS1), a key molecule of the classical negative feedback system that regulates cytokine signal transduction (59). In turn, when inflammation is chronically exacerbated, miR-155 overexpression attenuates inflammation intensity by targeting key TLR-signaling downstream molecules (60). Specifically, by targeting NF-κB p65, miR-155 overexpression has been shown to serve as a negative feedback regulator of inflammation, reducing TNF-α production (61, 62). Thus, we hypothesize that the upregulation of TNF-α levels in plasma and PBMCs of depression patients may result from a combined increase of miR-342, a TNF-α promoter, and downregulation of miR-155 and miR-146a, TNF-α negative regulators. Importantly, ROC analysis revealed that, when used in combination, the expression levels of miR-342, miR-146a and miR-155, constitute a diagnostic panel with increased sensibility and specificity. The combination of miRNAs in panels has been shown to increase their accuracy and diagnosis value, when compared to the use of single miRNAs (63).

Globally, we show that depression patients have increased systemic inflammation, reflected on increased plasma levels of TNF-α and CCL2, increased TNF-α mRNA levels in PBMCs, and dysregulated expression of key microglia and inflammation-related miRNAs in PBMCs. Future work should investigate the potential use of miR-342, miR-146a and miR-155 as a miRNA panel to diagnose depression and monitor treatment response*,* particularly in cases with exacerbated baseline inflammation.

Acknowledgments

Authors would like to thank Dr. José H. Teixeira and Dr. Andreia M. Silva and Sara R. Moura for their help with the samples processing. This work was funded by project NORTE-01-0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). JPB was supported by FCT- Fundação para a Ciência

e a Tecnologia, through BiotechHealth PhD program fellowship (PD/BD/135490/2018). MIA also thanks FCT (CEECINST/00091/2018/CP1500/CT0011).

References

1. Kessler RC, Bromet EJ. The epidemiology of depression across cultures. Annu Rev Public Health. 2013;34:119-38.
2. Vos T, Lim SS, Abbafati C, Abbas KM, Abbasi M, Abbasifard M, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. The Lancet. 2020;396(10258):1204-22.
3. Cipriani A, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y, et al. Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. The Lancet. 2018;391(10128):1357-66.
4. Blackburn TP. Depressive disorders: Treatment failures and poor prognosis over the last 50 years. Pharmacology research & perspectives. 2019;7(3):e00472.
5. Souery D, Oswald P, Massat I, Bailer U, Bollen J, Demyttenaere K, et al. Clinical factors associated with treatment resistance in major depressive disorder: results from a European multicenter study. The Journal of clinical psychiatry. 2007;68(7):1062-70.
6. Rush AJ, Trivedi MH, Wisniewski SR, Nierenberg AA, Stewart JW, Warden D, et al. Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. The American journal of psychiatry. 2006;163(11):1905-17.
7. Fried EI, Nesse RM. Depression sum-scores don't add up: why analyzing specific depression symptoms is essential. BMC medicine. 2015;13(1):72.
8. Osimo EF, Pillinger T, Rodriguez IM, Khandaker GM, Pariante CM, Howes OD. Inflammatory markers in depression: A meta-analysis of mean differences and variability in 5,166 patients and 5,083 controls. Brain, behavior, and immunity. 2020;87:901-9.
9. Brás JP, Pinto S, Almeida MI, Prata J, von Doellinger O, Coelho R, et al. Peripheral Biomarkers of Inflammation in Depression: Evidence from Animal Models and Clinical Studies. In: Kobeissy FH, editor. Psychiatric Disorders: Methods and Protocols: Springer; 2019. p. 467-92.
10. O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. Journal of psychiatric research. 2007;41(3-4):326-31.
11. Dantzer R, Kelley KW. Stress and immunity: an integrated view of relationships between the brain and the immune system. Life Sci. 1989;44(26):1995-2008.

12. Hart BL. Biological basis of the behavior of sick animals. Neuroscience and biobehavioral reviews. 1988;12(2):123-37.
13. Denicoff KD, Rubinow DR, Papa MZ, Simpson C, Seipp CA, Lotze MT, et al. The neuropsychiatric effects of treatment with interleukin-2 and lymphokine-activated killer cells. Annals of internal medicine. 1987;107(3):293-300.
14. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol. 2006;27(1):24-31.
15. Kim S, Foley FW, Picone MA, Halper J, Zemon V. Depression Levels and Interferon Treatment in People with Multiple Sclerosis. International Journal of MS Care. 2012;14(1):10-6.
16. Chiu WC, Su YP, Su KP, Chen PC. Recurrence of depressive disorders after interferon-induced depression. Translational psychiatry. 2017;7(2):e1026.
17. Udina M, Castellvi P, Moreno-Espana J, Navines R, Valdes M, Forns X, et al. Interferon-induced depression in chronic hepatitis C: a systematic review and meta-analysis. The Journal of clinical psychiatry. 2012;73(8):1128-38.
18. Köhler-Forsberg O, N. Lydholm C, Hjorthøj C, Nordentoft M, Mors O, Benros ME. Efficacy of anti-inflammatory treatment on major depressive disorder or depressive symptoms: meta-analysis of clinical trials. Acta psychiatrica Scandinavica. 2019;139(5):404-19.
19. Husain MI, Chaudhry IB, Khoso AB, Husain MO, Hodsoll J, Ansari MA, et al. Minocycline and celecoxib as adjunctive treatments for bipolar depression: a multicentre, factorial design randomised controlled trial. The Lancet Psychiatry. 2020;7(6):515-27.
20. Brites D, Fernandes A. Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation. Front Cell Neurosci. 2015;9:476.
21. Almeida MI, Reis RM, Calin GA. Decoy activity through microRNAs: the therapeutic implications. Expert opinion on biological therapy. 2012;12(9):1153-9.
22. Geaghan M, Cairns MJ. MicroRNA and Posttranscriptional Dysregulation in Psychiatry. Biological psychiatry. 2015;78(4):231-9.
23. Contreras J, Rao DS. MicroRNAs in inflammation and immune responses. Leukemia. 2012;26(3):404-13.
24. Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutation research. 2011;717(1-2):1-8.
25. McNeill E, Van Vactor D. MicroRNAs shape the neuronal landscape. Neuron. 2012;75(3):363-79.
26. Brás JP, Bravo J, Freitas J, Barbosa MA, Santos SG, Summavielle T, et al. TNF-alpha-induced microglia activation requires miR-342: impact on NF-κB signaling and neurotoxicity. Cell Death & Disease. 2020;11(6):415.

27. Schulte LN, Westermann AJ, Vogel J. Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing. Nucleic acids research. 2013;41(1):542-53.

28. Cardoso AL, Guedes JR, Pereira de Almeida L, Pedroso de Lima MC. miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. Immunology. 2012;135(1):73-88.

29. Li R, Shen Q, Wu N, He M, Liu N, Huang J, et al. MiR-145 improves macrophage-mediated inflammation through targeting Arf6. Endocrine. 2018;60(1):73-82.

30. Cai D, Wei D, Chen S, Chen X, Li S, Chen W, et al. MiR-145 protected the cell viability of human cerebral cortical neurons after oxygen-glucose deprivation by downregulating EPHA4. Life Sci. 2019;231:116517.

31. Fregeac J, Moriceau S, Poli A, Nguyen LS, Oury F, Colleaux L. Loss of the neurodevelopmental disease-associated gene miR-146a impairs neural progenitor differentiation and causes learning and memory deficits. Molecular Autism. 2020;11(1):22.

32. Chen L, Dong R, Lu Y, Zhou Y, Li K, Zhang Z, et al. MicroRNA-146a protects against cognitive decline induced by surgical trauma by suppressing hippocampal neuroinflammation in mice. Brain, behavior, and immunity. 2019;78:188-201.

33. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.

34. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nat Rev Immunol. 2016;16(1):22-34.

35. Leighton SP, Nerurkar L, Krishnadas R, Johnman C, Graham GJ, Cavanagh J. Chemokines in depression in health and in inflammatory illness: a systematic review and meta-analysis. Molecular psychiatry. 2018;23(1):48-58.

36. Yuan N, Chen Y, Xia Y, Dai J, Liu C. Inflammation-related biomarkers in major psychiatric disorders: a cross-disorder assessment of reproducibility and specificity in 43 meta-analyses. Translational psychiatry. 2019;9(1):233.

37. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A meta-analysis of cytokines in major depression. Biological psychiatry. 2010;67(5):446-57.

38. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience. 2008;9(1):46-56.

39. Felger JC, Lotrich FE. Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. Neuroscience. 2013;246:199-229.

40. Liew C-C, Ma J, Tang H-C, Zheng R, Dempsey AA. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. Journal of Laboratory and Clinical Medicine. 2006;147(3):126-32.

41. Hasselmann H, Gamradt S, Taenzer A, Nowacki J, Zain R, Patas K, et al. Pro-inflammatory Monocyte Phenotype and Cell-Specific Steroid Signaling Alterations in Unmedicated Patients With Major Depressive Disorder. Frontiers in Immunology. 2018;9(2693).

42. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. The New England journal of medicine. 2005;353(17):1793-801.

43. Egeland NG, Lunde S, Jonsdottir K, Lende TH, Cronin-Fenton D, Gilje B, et al. The Role of MicroRNAs as Predictors of Response to Tamoxifen Treatment in Breast Cancer Patients. Int J Mol Sci. 2015;16(10):24243-75.

44. Singh PK, Preus L, Hu Q, Yan L, Long MD, Morrison CD, et al. Serum microRNA expression patterns that predict early treatment failure in prostate cancer patients. Oncotarget. 2014;5(3):824-40.

45. Yirmiya R, Rimmerman N, Reshef R. Depression as a microglial disease. Trends in neurosciences. 2015;38(10):637-58.

46. D'Mello C, Le T, Swain MG. Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29(7):2089-102.

47. Walker FR, Nilsson M, Jones K. Acute and chronic stress-induced disturbances of microglial plasticity, phenotype and function. Current drug targets. 2013;14(11):1262-76.

48. Haroon E, Miller AH. Inflammation Effects on Brain Glutamate in Depression: Mechanistic Considerations and Treatment Implications. Current topics in behavioral neurosciences. 2017;31:173-98.

49. Lu Q, Wu R, Zhao M, Garcia-Gomez A, Ballestar E. miRNAs as Therapeutic Targets in Inflammatory Disease. Trends in Pharmacological Sciences. 2019;40(11):853-65.

50. Saba R, Sorensen DL, Booth SA. MicroRNA-146a: A Dominant, Negative Regulator of the Innate Immune Response. Frontiers in Immunology. 2014;5(578).

51. Meisgen F, Xu Landén N, Wang A, Réthi B, Bouez C, Zuccolo M, et al. MiR-146a Negatively Regulates TLR2-Induced Inflammatory Responses in Keratinocytes. Journal of Investigative Dermatology. 2014;134(7):1931-40.

52. Wang J, Yan Y, Song D, Liu B. Reduced Plasma miR-146a Is a Predictor of Poor Coronary Collateral Circulation in Patients with Coronary Artery Disease. BioMed research international. 2016;2016:4285942.

53. Chen BB, Li ZH, Gao S. Circulating miR-146a/b correlates with inflammatory cytokines in COPD and could predict the risk of acute exacerbation COPD. Medicine. 2018;97(7):e9820.
54. Tang Y, Luo X, Cui H, Ni X, Yuan M, Guo Y, et al. MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. Arthritis and rheumatism. 2009;60(4):1065-75.
55. Smalheiser NR, Lugli G, Rizavi HS, Torvik VI, Turecki G, Dwivedi Y. MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects. PloS one. 2012;7(3):e33201-e.
56. Hung Y-Y, Wu M-K, Tsai M-C, Huang Y-L, Kang H-Y. Aberrant Expression of Intracellular let-7e, miR-146a, and miR-155 Correlates with Severity of Depression in Patients with Major Depressive Disorder and Is Ameliorated after Antidepressant Treatment. Cells. 2019;8(7):647.
57. Mahesh G, Biswas R. MicroRNA-155: A Master Regulator of Inflammation. J Interferon Cytokine Res. 2019;39(6):321-30.
58. Woodbury ME, Freilich RW, Cheng CJ, Asai H, Ikezu S, Boucher JD, et al. miR-155 Is Essential for Inflammation-Induced Hippocampal Neurogenic Dysfunction. The Journal of Neuroscience. 2015;35(26):9764-81.
59. Pathak S, Grillo AR, Scarpa M, Brun P, D'Incà R, Nai L, et al. MiR-155 modulates the inflammatory phenotype of intestinal myofibroblasts by targeting SOCS1 in ulcerative colitis. Experimental & molecular medicine. 2015;47(5):e164-e.
60. O'Neill LA, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat Rev Immunol. 2011;11(3):163-75.
61. Wu XY, Fan WD, Fang R, Wu GF. Regulation of microRNA-155 in endothelial inflammation by targeting nuclear factor (NF)-κB P65. Journal of cellular biochemistry. 2014;115(11):1928-36.
62. Li X, Kong D, Chen H, Liu S, Hu H, Wu T, et al. miR-155 acts as an anti-inflammatory factor in atherosclerosis-associated foam cell formation by repressing calcium-regulated heat stable protein 1. Scientific Reports. 2016;6(1):21789.
63. Wang C, Ding M, Xia M, Chen S, Van Le A, Soto-Gil R, et al. A Five-miRNA Panel Identified From a Multicentric Case–control Study Serves as a Novel Diagnostic Tool for Ethnically Diverse Non-small-cell Lung Cancer Patients. EBioMedicine. 2015;2(10):1377-85.

Supplementary Information

A clinical study of inflammatory and miRNA mediators in depression

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Supplementary Table 1. Oligonucleotides sequences for RT-qPCR. Oligonucleotides used to amplify human mRNAs encoding inflammatory markers and reference gene, based on GenBank sequences. Abbreviations: IL1B, interleukin 1 beta; IL6, interleukin 6; TNF, tumour necrosis factor; CCL2, C-C motif chemokine ligand 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Gene	Accession Number	Forward (5'-3')	Reverse (5'-3')
IL1B	NM_000576.3	CTTCAGCCAATCTTCATT	CACTGTAATAAGCCATCAT
IL6	NM_000600.5	AATTCGGTACATCCTCGACGG	GGTTGTTTTCTGCCAGTGCC
TNF	NM_000594.4	TCTCTCTAATCAGCCCTCTG	TGCTACAACATGGGCTACAG
CCL2	NM_002982.4	CAGCCAGATGCAATCAATGC	GCACTGAGATCTTCCTATTGGTGAA
GAPDH	NM_002046.7	CCATCCACAGTCTTCTGGGT	CCTCAAGATCATCAGCAAT

Supplementary Table 2. Spearman correlations between plasma cytokines in depression patients. The coefficient r and *p*-value for each correlation are presented. Statistically significant correlations are highlighted (p<0.05).

		Plasma											
		CCL2		IL-6		IL-1β		TNF-α		IL-4		IL-10	
		r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
Plasma	**CCL2**	-	-	**0.5925**	***0.0003***	-0.0152	*0.9340*	0.3171	*0.0769*	**0.3940**	***0.0256***	**0.4801**	***0.0256***
	IL-6	-	-	-	-	-0.1613	*0.3776*	0.2648	*0.1428*	**0.4917**	***0.0042***	**0.7326**	***1.8x10^{-6}***
	IL-1β	-	-	-	-	-	-	0.0819	*0.6558*	0.0513	*0.7800*	0.2185	*0.2294*
	TNF-α	-	-	-	-	-	-	-	-	0.2552	*0.1585*	0.2552	*0.7503*
	IL-4	-	-	-	-	-	-	-	-	-	-	**0.4929**	***0.0041***
	IL-10	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Table 3. Spearman correlations between cytokines mRNA levels in PBMCs of depression patients. The coefficient r and *p*-value for each correlation are presented. Statistically significant correlations are highlighted (p<0.05).

		PBMCs							
		CCL2		IL-6		IL-1β		TNF-α	
		r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
PBMCs	**CCL2**	-	-	**0.4872**	***0.0214***	-0.0762	*0.7359*	**0.6374**	***0.0014***
	IL-6	-	-	-	-	-0.1326	*0.5560*	**0.5629**	***0.0063***
	IL-1β	-	-	-	-	-	-	0.0818	*0.7171*
	TNF-α	-	-	-	-	-	-	-	-

Supplementary Table 4. Spearman correlations between miRNA levels in PBMCs of depression patients. The coefficient r and *p*-value for each correlation are presented. Statistically significant correlations are highlighted (p<0.05).

		PBMCs							
		miR-342		miR-145		miR-146a		miR-155	
		r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
PBMCs	**miR-342**	-	-	0.0028	*0.9879*	0.2584	*0.1603*	0.2552	*0.1658*
	miR-145	-	-	-	-	-0.0935	*0.6166*	0.0193	*0.9176*
	miR-146a	-	-	-	-	-	-	0.2737	*0.1361*
	miR-155	-	-	-	-	-	-	-	-

Supplementary Table 5. ROC analysis of the differently expressed miRNAs, individually or combined. AUC – area under the curve; CI – confidence interval.

miRNA Panel	miRNAs	AUC	Std. Error	CI	*p-value*
-	**miR-342**	0.6669	0.0698	0.53-0.80	0.0164
-	**miR-146a**	0.7363	0.06018	0.62-0.85	0.0007
-	**miR-155**	0.6911	0.06257	0.57-0.81	0.006
A	**miR-342 + miR-146a**	0.8065	0.05234	0.70-0.90	<0.0001
B	**miR-342 + miR-155**	0.7629	0.05876	0.65-0.88	0.0002
C	**miR-146a + miR-155**	0.7411	0.05797	0.63-0.85	0.0005
D	**miR-342 + miR-146a + miR-155**	0.8419	0.04679	0.75-0.93	<0.0001

III. General discussion and future perspectives

The contributions of the immune system, cells and mediators, for depression pathophysiology are increasingly recognized. However, many details of the crosstalk between immune and nervous systems remain unclear. For this doctoral thesis we focused on the identification and validation of ncRNAs, particularly miRNAs, as regulators of microglia activation, a feature of inflammation-related depression, and evaluated their potential as biomarkers. Our studies identified miR-342, a TNF-α-induced miRNA involved in microglia activation, that appeared dysregulated in the brain of depressive rats and in PBMCs of subjects diagnosed with major depression.

Depression ranks as the most prevalent psychiatric disorder and one of the leading causes of disability worldwide (1). Conventional pharmacological treatments for depression mainly target neurotransmission regulation (2). However, due to the multifactorial aetiology of this disorder, it is estimated at least one third of the patients do not respond to one or several antidepressant medication schemes (3-5). Therefore, increasing efforts have been made to understand the different pathological mechanisms underlying depression in order to find alternative diagnosis and prognosis biomarkers, as well as to develop a wider spectrum of novel therapeutics. In this sense, inflammation has emerged as a key player in depression symptomatology and treatment resistance (6-8), for several reasons, including: 1) patients with chronic inflammatory diseases have a much higher prevalence of depression (9-13); 2) most treatment resistant patients present a hyper-activation of the immune system (14-16); 3) a segment of depression patients present chronic low-grade systemic and brain inflammation, characterized by increased levels of TNF-α, IL-6 and IL-1β (15, 17-20), trafficking of immune cells to the brain, and activation of microglia (21, 22). Although the contribution of inflammation to the worsening of depression is now established, the use of anti-inflammatory drugs as efficient antidepressants (23), and the anti-inflammatory properties of traditional antidepressants, remain a subject of intense debate (24-27). The reasoning for that lies with our limited knowledge of the mechanisms underlying most of the findings reported in those observational studies. These molecular mechanisms need to be further explored and explained. Moreover, specific mediators that may link inflammation and depression remain to be identified, validated and tested as potential biomarkers and/or therapeutical targets. In this particular aspect, miRNAs have shown potential as target molecules for diagnosis and/or treatment, due to their involvement in important disease-associated mechanisms. Due to their multi-targeted regulatory role, miRNAs may provide answers to the integration of the inflammatory process and depressive symptoms. Therefore, the main goal of this PhD thesis was to understand how miRNAs could concomitantly impact and integrate both processes.

Initially, we explored the role of miRNAs in microglia activation and in microglia-to-neurons communication. Microglia are the innate immune cells of the brain playing series of physiological roles, ranging from removal of neuronal debris to precise refinement of synaptic

terminals, contributing to maturation of neural circuits (28). Microglia are very dynamic, constantly engaged in physical contacts with neighbouring neurons and other glial cells (namely astrocytes and oligodendrocytes) (29). When an injury or infection takes place, microglia are recruited to the site where they engulf invading pathogens and extracellular debris by phagocytosis, supporting the normal function and integrity of the brain (30). In response to an inflammatory stimulus, microglia secrete a number of molecules such as proteinases, nitric oxide, reactive oxygen intermediates and pro-inflammatory cytokines (21, 31). However, in pathological conditions, such as in chronic inflammation-related depression, in which pro-inflammatory cytokine levels are elevated and microglia become hyperactivated and dysfunctional, shifting away from performing homeostatic functions, rather causing neuronal damage and degeneration, thereby affecting normal neurocircuits. Hyperactivated microglia are particularly characterized by overproduction of TNF-α that can be perpetuated by a positive feedback mechanism of autocrine activation (32). In physiological conditions, TNF-α has important roles in brain development and host defence responses (33), but a chronic increase in TNF-α levels is known to have deleterious effects on brain cells and neurocircuits (34) (35-37). However, specific molecular mediators that can be effectively targeted to control TNF-α-mediated microglia overactivation, are yet to be uncovered.

Therefore, in Chapter 2.1 we evaluated whether TNF-α induced microglia activation would influence miRNA expression profiles. As expected, TNF-α stimulation induced an upregulation of pro-inflammatory markers NF-κB, Nos2, IL-1β and TNF-α. The miRNA microarray revealed a group of miRNAs that were dysregulated compared with resting microglia. Further validation identified, for the first time, miR-342 as being significantly upregulated in response to TNF-α activation. However, although they were not pursued in the current work, other miRNAs stood out from the miRNA microarray and may deserve further investigation. On one hand, miR-146b and miR-181b known to be upregulated in response to NF-κB activation, figured among the most upregulated miRNAs, in what we hypothesize to be a negative feedback mechanism to control excessive cell activation, as described in Chapter 1.1.3. Also, miR-494, upregulated in TNF-α activated microglia, has been described to potentiate pro-inflammatory responses and to be dysregulated in depression patients at baseline and in response to treatment (38, 39). On the contrary miR-124, miR-9a and several members of the let-7 family were found downregulated. All these miRNAs are known to have important anti-inflammatory roles, and we hypothesize that their downregulation is associated with a mechanism that cells use during the acute response to TNF-α, thereby favouring the expression of a set of miRNAs that promote a pro-inflammatory activation over inhibitory miRNAs. This is in line with previous work from our group in an unrelated injury model, in bone, where we also showed a temporal regulation of several members of the let-7 family, with down-regulation at day 3 (acute inflammation) and up-regulation at day 14 (inflammation resolution) (40).

Nonetheless, miR-342 was the only miRNA consistently upregulated in multiple independent experiments. At that time, no specific roles in inflammation or microglia activation had been described for this miRNA, and for that reason we further investigated its involvement in TNF-α driven microglia activation. We found that miR-342 overexpression *per se* induced microglia activation by promoting NF-κB activation and increased secretion of TNF-α and IL-1β. Conversely, miR-342 inhibition led to a strong decrease in the levels of these cytokines after TNF-α activation, supporting a crucial role for this miRNA during TNF-α-driven microglia activation. To investigate potential miR-342 targets, we hypothesized that this miRNA could be repressing an inhibitor of the NF-κB pathway. Proteomics analysis revealed several proteins affected by the overexpression of miR-342, of which BAG-1, that had been previously described to promote NF-κB p65 degradation in LPS-treated dendritic cells (41), was significantly downregulated. BAG-1 (BCL-2 associated athanogene 1) is a widely expressed protein that interacts with a number of signaling molecules, including BCL-2 and Raf-1, thereby regulating pathways involved in cell activation, survival and differentiation (42). The most common role played by BAG-1 protein is as an inhibitor of apoptosis, but there are now increasing evidences of its contribution as mediator of cell activation in inflammation (41, 43). Nonetheless, it is important to note that although the inhibitory effect of the BAG-1 overexpression over NF-κB p65 was confirmed, we cannot guarantee that BAG-1 is a direct target of miR-342. miR-342 overexpression was shown to drastically inhibit BAG-1 expression, however *in silico* analysis revealed no predicted target sites for an interaction between miR-342 and BAG-1. Therefore, we hypothesize that the regulatory effect produced by miR-342 over BAG-1 may be occurring through the inhibition of a BAG-1 upstream regulator, that should be further investigated. Also, we do not exclude that other proteins with relevance for microglia activation, can also be affected by miR-342 overexpression. DAVID functional annotation analysis revealed that inflammation-related processes were the most upregulated biological functions associated with miR-342 overexpression and therefore other protein candidates may be potential targets. Next, to evaluate whether miR-342 overexpression on microglia would impact their crosstalk and potentially become harmful to neurons, we co-cultured microglia and hippocampal neurons in a microfluidic system that allowed direct contact between microglia and neuronal axons. We found that miR-342 overexpression in microglia significantly affected neuron viability to levels similar of those produced by TNF-α overactivation. Of note, increased levels of nitrites were detected in the supernatants of these co-cultures. As mentioned before, when activated, microglia not only go through series of transcriptional changes that lead to the production of pro-inflammatory cytokines, but also neurotoxic effectors, such as nitric oxide (44, 45). Thus, with the work developed in this task we identified miR-342 as a new potential target to resolve neuroinflammation, characterized

by increased levels of TNF-α and sustained microglia activation, that are critical features of inflammation-related depression.

However, despite these promising findings, it remained to be elucidated if miR-342 would actually be dysregulated and associated with depression and inflammation *in vivo*, particularly in the brain and systemically. Thus, this was investigated in the following two Chapters, using first an animal model of depression, and then in a clinical cohort of depression patients.

In Chapter 2.2 we used rat model in collaboration with Prof. Carmen Sandi's Lab, with differential CSR and PPS as triggers of depression. The peripubertal period, comprising childhood and adolescence, is a critical time window in brain development that is sensitive to the deleterious effects of adverse experiences, enhancing the risk of developing depression in adulthood (46-48). PPS in rats was shown to enhance anxiety-related behaviours and increased passive stress coping responses in adulthood, a key symptom in depression (49, 50). Glucocorticoids, especially CORT in rodents (equivalent to cortisol in humans), coordinate responses that enable an individual to cope with stressful challenges, mediating adaptation following a stressor's cessation (51). However, there is substantial individual variability in the magnitude of glucocorticoid responsiveness to stressors, a trait highly related to differences in coping styles (52, 53). In this sense, Carmen Sandi's Lab developed a selective breeding protocol that generates lines of rats enriched for different levels of CSR (54). Thus, the choice of this model was based on the attempt to understand if the exposure to risk factors that naturally increase the susceptibility to develop depression would also induce neuroinflammation, rather than an approach that directly induces exacerbated systemic and local inflammation, such as LPS administration.

In fact, there are increasing evidences indicating that stress and dysfunctional HPA responses, can lead to the activation of inflammatory responses in the brain as well as peripherally (55, 56). When stress is under control, the body responds in a physiological way by activating the HPA axis that normally suppresses the immune system through the release of glucocorticoids. Glucocorticoids coordinate responses that enable an individual to cope with stressful challenges, mediating adaptation following a stressor's cessation, helping to maintain stability through change, in a process called allostasis (57). However, when stress becomes chronic, overexaggerated or occurs in temporal windows critical for brain development (e.g., in youth), it leads to a downregulation of glucocorticoid receptors involved in the negative feedback controlling the HPA axis resulting in the failure to suppress inflammatory responses (58). As such, elevated pro-inflammatory cytokines, increased microglia activation and accumulation of peripherally derived monocytes and macrophages have been observed in the brain following stress exposure (55).

Thus, in this Chapter we also aimed to investigate the long-term effect of PPS and high-CSR (H-CSR) in male rats in terms of depressive-like behaviours, inflammatory markers

across different brain regions, microglial activation and miR-342 expression. We found that H-CSR male rats that underwent PPS exhibited depressive-like behaviours such as reduced sociability, increased anhedonia and passive coping responses in adulthood. This suggests that individuals with constitutive H-CSR are particularly sensitive to developing protracted depression-like behaviours following early-life stress exposure. Interestingly, although a chronic increase in CORT was previously reported to be sufficient to induce depression-like behaviours in rats (59), we found that H-CSR alone, without priming by stressful events, was not sufficient to induce depressive-like behaviours in rats. This can be explained by the fact that in our model, animals do not have persistently increased levels of CORT, but rather a natural exacerbated CORT production in response to stress exposures. In fact, a wide consensus has been reached towards the fact that increased incidence of depression in humans is associated with greater cortisol response variability rather than higher baseline levels of cortisol (52).

Evaluation of brain inflammatory markers across different brain regions known to be involved in the pathophysiology of depression, revealed a marked increase of TNF-α and IBA-1 in the hippocampus of PPS H-CSR rats. In addition, these animals also exhibited late effects on hippocampal microglia, with morphological alterations compatible with a higher degree of activation. Specifically, hippocampal TNF-α expression and microglia activation in adulthood tend to be positively correlated with passive coping responses in adulthood, as determined by FST. Interestingly, a recent review on the neuro-immunological short-term and long-term effects of maternal separation in rodents, revealed short-term greater microglial activation and elevated pro-inflammatory cytokine signalling in key brain regions implicated in human psychiatric disorders, but generally no long-term effect on cytokine expression in the absence of later-life stress (60). Conversely, our findings indicate that PPS produced long-term neuro-immunological alterations in H-CSR rats, particularly in the hippocampus. Although the evaluation of the short-term effect of PPS may provide new insights on this issue, we hypothesize that the temporal window in which the animals were subjected to those extreme stressful conditions (peripubertal period, P28 - P42), might influence the long-term effect. PPS covers different periods throughout the juvenile and pubertal developmental stages in the rat and has been shown to lead to increased emotionality, decreased sociability and pathological aggression (46). Tzanoulinou *et al.* reported that the full extent of the PPS protocol is required for the observed behavioural and neurobiological effects, because exposure corresponding only to the period of male rats childhood/prepuberty (P28–P30) or puberty (P40 - P42) is insufficient to elicit the same effects (61). Also, by having differential CORT responses to stress in our experimental model, we were able better analyse the impact of individual differences in regulation of glucocorticoid response to repeated exposure to stressful

challenges, which has been associated with different vulnerabilities to develop psychopathologies, including depression (52).

A limitation of this study, and a question to address in the future, is that we focused in males, while depression in humans is more frequent in females (62), but severe suicide attempts are more frequent in males (63). Nonetheless, sex-specific mechanisms have been shown to regulate response to stress and the development of depression outcomes (64). Also, as discussed in Chapter 2.2, since separate hemispheres were used to perform gene expression and microglia morphological analysis, we cannot exclude any laterality effect. Moreover, dysregulation of microglial activation and cytokine expression levels may be also occurring in brain regions other than those studied. Therefore, future studies should also compare and clarify whether stressful events at different stages of brain development may have distinct long-term effects in terms of neuroinflammation across different brain regions, and how it may correlate with depressive-like behaviours in both males and females.

Following our findings of increased microglia activation and TNF-α levels in the hippocampus of animals exhibiting depressive-like behaviours, we tested our main hypothesis, on whether miR-342 could be dysregulated and correlated with these observations. We found that H-CSR animals that underwent PPS showed upregulation of hippocampal miR-342 and its expression was positively correlated with TNF-α expression, microglial activation and passive coping responses. Strikingly, these results corroborated, *in vivo*, our previous findings, of a strong interplay between miR-342, TNF-α and microglial activation. To further validate this interdependent mechanism *in vivo*, future research should provide evidences on whether microglia are the main source of TNF-α and miR-342 is specifically overexpressed in microglia and/or is also overexpressed in other brain cell types or in extracellular vesicles. Also, in the future, manipulation of miR-342 levels in the hippocampus will provide objective data on the functional implications of this miRNA in microglia activation, TNF-α levels and resulting behavioural implications. For instance, overexpression of miR-301b was recently shown to accelerate hippocampal microglia activation and cognitive impairment in mice with depressive-like behaviour through the NF-κB signalling pathway (65). Altogether our findings identified, for the first time, miR-342 as a novel potential therapeutic target for neuroinflammation in the hippocampus and as a potential biomarker for inflammation-related depression.

Finally, in order to explore the potential of inflammation-related miRNAs, particularly miR-342, as peripheral diagnostic markers of depression, a proof-of-concept trial in a cohort of 72 subjects (32 depression patients and 40 healthy controls) was performed. The levels of inflammatory cytokines in plasma and PBMCs and the expression levels of inflammation-related miRNAs in PBMCs were evaluated. We found increased levels of TNF-α and CCL2 in the plasma of depression patients, while no differences were found for IL-6, IL-1β, IL-4 and

IL-10. On the other hand, mRNA levels of TNF-α were found upregulated and IL-6 mRNA levels significantly downregulated in PBMCs of depression patients compared with healthy subjects. Globally these results point towards a low-grade chronic inflammatory state rather than an exacerbated increased of several inflammatory markers. In agreement, multiple reports on this issue have been reporting low-grade inflammation in depression patients (14, 66). Functional implications of low-grade inflammation were also recently shown in context of post-traumatic stress disorder (PTSD), where alongside with stress, render individuals more vulnerable to PTSD (67).

We then sought to correlate this with the expression of inflammation-related miRNAs in PBMCs. Interestingly, miR-342 levels were found upregulated, while miR-146a and miR-155 were significantly downregulated, and miR-145 did not differ between depression patients and healthy controls. To our knowledge, this is the first study reporting increased expression levels of miR-342 in PBMCs of depression patients. Importantly, miR-342 levels were positively correlated with TNF-α levels in PBMCs, showing that a strong interplay between TNF-α and miR-342 is also translated to the periphery of human depression patients. Conversely, miR-146a and miR-155 were found downregulated. In fact, mounting evidences suggest that miR-146a is downregulated in pathologies with baseline low-grade chronic inflammation, such as hypertension and obesity, as referred in section 1.3.2 (68, 69). Moreover, our study corroborated the described role of miR-146 family in inflammation. On one hand, the miRNA microarray showed that miR-146b was upregulated in microglia in the acute response to TNF-α and NF-κB activation. On the other hand, in a situation of chronic low-grade inflammation miR-146a was found downregulated in PBMCs of depression patients. Regarding miR-155, while previous studies have reported increased serum levels of this miRNA in depression patients, and an association between its upregulation in the CNS and acute microglia-mediated inflammatory responses (70, 71), our current findings of low expression of miR-155 in PBMCs are not in agreement with those studies. In line with our findings, a recent study performed by Hung *et al.,* evaluating the expression levels of intracellular miRNAs that regulate TLR4 signaling in PBMCs and monocytes of depression patients, also found a downregulation of intracellular miR-146a and miR-155 in PBMCs (57). Although the authors did not evaluate the levels of inflammatory markers, these findings support the idea of an aberrant expression of inflammation-associated miRNAs in depression patients, that may be related with a dysfunction of immune cells due to chronic inflammation. Similarly to miR-146a, miR-155 was recently shown to be induced by TNF-α, which in turn suppresses excessive TNF-α production in order resolve inflammation (72). However, in the current study, we hypothesize that the increase of TNF-α detected in plasma and PBMCs, has been perpetuated for some time in lower levels than would be expected for a typical acute inflammation. These chronically increased levels of TNF-α likely produced changes in innate immune cells

reactivity, that are reflected in aberrant transcriptional profiles, as previously shown by others (73). Globally, we therefore hypothesize that the upregulation of TNF-α levels in plasma and PBMCs of depression patients may result from a combined increase of miR-342, a TNF-α promoter, and a downregulation of miR-155 and miR-146a, TNF-α negative regulators.

Finally, we explored the potential of the differently expressed miRNAs to serve as peripheral diagnostic markers of depression by testing their ability to distinguish between depression patients and healthy controls, individually or in combination. ROC analysis revealed that, when tested as a miRNA panel, the use of the three miRNAs in combination was the best classifier, with potential to constitute a good diagnostic panel. These promising results should now be further validated in PBMCs and plasma of a larger cohort of patients, evaluating their potential for diagnosis and better patient stratification, in terms of depression severity, age, sex, inflammatory profile and other comorbidities. Also, their potential to be used as monitors of treatment response should be evaluated in the future.

The work developed in this thesis aimed to build up knowledge into the role of miRNAs in regulating inflammation-related depression and its underlying mechanisms. Taken together, the results presented and discussed herein identify miR-342 as a potential therapeutic target to promote neuroinflammation resolution. We described the mechanism by which it contributes to TNF-α driven microglia activation, and found it upregulated in the hippocampus of adult rats with early life stress-induced depressive-like behaviours and increased neuroinflammation (Figure 1). Finally, miR-342 was found upregulated in PBMCs of human patients diagnosed with depression, and together with miR-146a and miR-155 was shown to constitute a potential miRNA biomarker panel for depression diagnosis (Figure 1). Of note, a common thread to both the *in vivo* and the proof-of-concept clinical trial was a strong correlation between miR-342 and TNF-α levels. Therefore, future studies should explore if miR-342 may be also dysregulated in other pathologies with increased baseline inflammation, particularly TNF-α levels, as well as in other psychiatric disorders. Moreover, modulation of miR-342 levels as a therapeutical approach to improve depression symptomatology should be tested. These findings will help to clarify its potential as a specific diagnostic marker and therapeutical target for inflammation-related depression.

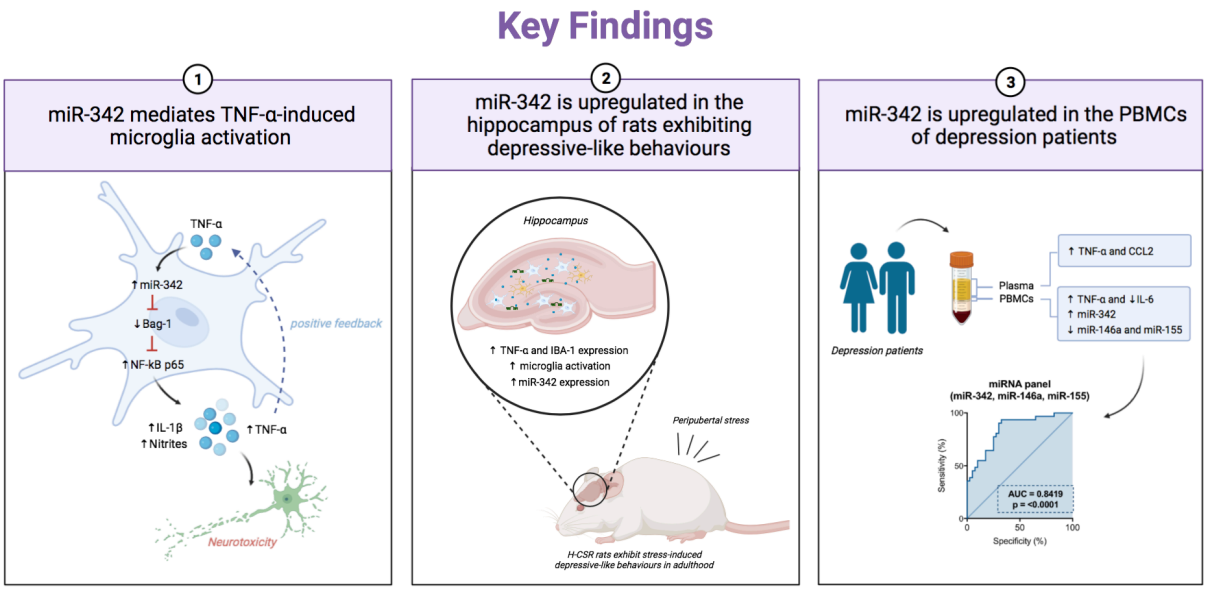

Figure 1. miR-342 as a potential biomarker and therapeutical target for inflammation-related depression. (1) TNF-α-induced miR-342 promotes microglia activation through NF-κB and induces neurotoxicity; (2) Stress-induced depressive-like behaviours in male rats are associated with miR-342 upregulation and neuroinflammation in the hippocampus; (3) miR-342 is upregulated in the PBMCs of depression patients and, together with miR-146a and miR-155, have the potential to constitute a good diagnostic panel.

References

1. Vos T, Lim SS, Abbafati C, Abbas KM, Abbasi M, Abbasifard M, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. The Lancet. 2020;396(10258):1204-22.
2. Cipriani A, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y, et al. Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. The Lancet. 2018;391(10128):1357-66.
3. Blackburn TP. Depressive disorders: Treatment failures and poor prognosis over the last 50 years. Pharmacology research & perspectives. 2019;7(3):e00472.
4. Souery D, Oswald P, Massat I, Bailer U, Bollen J, Demyttenaere K, et al. Clinical factors associated with treatment resistance in major depressive disorder: results from a European multicenter study. The Journal of clinical psychiatry. 2007;68(7):1062-70.
5. Rush AJ, Trivedi MH, Wisniewski SR, Nierenberg AA, Stewart JW, Warden D, et al. Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. The American journal of psychiatry. 2006;163(11):1905-17.
6. Krishnan V, Nestler EJ. The molecular neurobiology of depression. Nature. 2008;455(7215):894-902.
7. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nat Rev Immunol. 2016;16(1):22-34.
8. Miller AH. Beyond depression: the expanding role of inflammation in psychiatric disorders. World Psychiatry. 2020;19(1):108-9.
9. Denicoff KD, Rubinow DR, Papa MZ, Simpson C, Seipp CA, Lotze MT, et al. The neuropsychiatric effects of treatment with interleukin-2 and lymphokine-activated killer cells. Annals of internal medicine. 1987;107(3):293-300.
10. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol. 2006;27(1):24-31.
11. Kim S, Foley FW, Picone MA, Halper J, Zemon V. Depression Levels and Interferon Treatment in People with Multiple Sclerosis. International Journal of MS Care. 2012;14(1):10-6.
12. Chiu WC, Su YP, Su KP, Chen PC. Recurrence of depressive disorders after interferon-induced depression. Translational psychiatry. 2017;7(2):e1026.
13. Udina M, Castellvi P, Moreno-Espana J, Navines R, Valdes M, Forns X, et al. Interferon-induced depression in chronic hepatitis C: a systematic review and meta-analysis. The Journal of clinical psychiatry. 2012;73(8):1128-38.

14. Osimo EF, Pillinger T, Rodriguez IM, Khandaker GM, Pariante CM, Howes OD. Inflammatory markers in depression: A meta-analysis of mean differences and variability in 5,166 patients and 5,083 controls. Brain, behavior, and immunity. 2020;87:901-9.

15. Brás JP, Pinto S, Almeida MI, Prata J, von Doellinger O, Coelho R, et al. Peripheral Biomarkers of Inflammation in Depression: Evidence from Animal Models and Clinical Studies. In: Kobeissy FH, editor. Psychiatric Disorders: Methods and Protocols: Springer; 2019. p. 467-92.

16. O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. Journal of psychiatric research. 2007;41(3-4):326-31.

17. Fitzgerald P, O'Brien SM, Scully P, Rijkers K, Scott LV, Dinan TG. Cutaneous glucocorticoid receptor sensitivity and pro-inflammatory cytokine levels in antidepressant-resistant depression. Psychological medicine. 2006;36(1):37-43.

18. Harrison NA, Brydon L, Walker C, Gray MA, Steptoe A, Critchley HD. Inflammation causes mood changes through alterations in subgenual cingulate activity and mesolimbic connectivity. Biological psychiatry. 2009;66(5):407-14.

19. Lanquillon S, Krieg JC, Bening-Abu-Shach U, Vedder H. Cytokine production and treatment response in major depressive disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2000;22(4):370-9.

20. Sluzewska A, Sobieska M, Rybakowski JK. Changes in acute-phase proteins during lithium potentiation of antidepressants in refractory depression. Neuropsychobiology. 1997;35(3):123-7.

21. Dheen ST, Kaur C, Ling EA. Microglial activation and its implications in the brain diseases. Current medicinal chemistry. 2007;14(11):1189-97.

22. Yirmiya R, Rimmerman N, Reshef R. Depression as a microglial disease. Trends in neurosciences. 2015;38(10):637-58.

23. Husain MI, Chaudhry IB, Khoso AB, Husain MO, Hodsoll J, Ansari MA, et al. Minocycline and celecoxib as adjunctive treatments for bipolar depression: a multicentre, factorial design randomised controlled trial. The Lancet Psychiatry. 2020;7(6):515-27.

24. Hiles SA, Baker AL, de Malmanche T, Attia J. Interleukin-6, C-reactive protein and interleukin-10 after antidepressant treatment in people with depression: a meta-analysis. Psychological medicine. 2012;42(10):2015-26.

25. Sluzewska A, Rybakowski JK, Laciak M, Mackiewicz A, Sobieska M, Wiktorowicz K. Interleukin-6 serum levels in depressed patients before and after treatment with fluoxetine. Annals of the New York Academy of Sciences. 1995;762:474-6.

26. Jazayeri S, Keshavarz SA, Tehrani-Doost M, Djalali M, Hosseini M, Amini H, et al. Effects of eicosapentaenoic acid and fluoxetine on plasma cortisol, serum interleukin-1beta

and interleukin-6 concentrations in patients with major depressive disorder. Psychiatry research. 2010;178(1):112-5.

27. Kubera M, Kenis G, Bosmans E, Kajta M, Basta-Kaim A, Scharpe S, et al. Stimulatory effect of antidepressants on the production of IL-6. International immunopharmacology. 2004;4(2):185-92.

28. Basilico B, Pagani F, Grimaldi A, Cortese B, Di Angelantonio S, Weinhard L, et al. Microglia shape presynaptic properties at developing glutamatergic synapses. Glia. 2019;67(1):53-67.

29. Thion MS, Ginhoux F, Garel S. Microglia and early brain development: An intimate journey. Science. 2018;362(6411):185-9.

30. Ransohoff RM, Perry VH. Microglial physiology: unique stimuli, specialized responses. Annual review of immunology. 2009;27:119-45.

31. Perry VH, Holmes C. Microglial priming in neurodegenerative disease. Nat Rev Neurol. 2014;10(4):217-24.

32. Probert L. TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. Neuroscience. 2015;302:2-22.

33. Golan H, Levav T, Mendelsohn A, Huleihel M. Involvement of tumor necrosis factor alpha in hippocampal development and function. Cerebral cortex (New York, NY : 1991). 2004;14(1):97-105.

34. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience. 2008;9(1):46-56.

35. Olmos G, Llado J. Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. Mediators of inflammation. 2014;2014:12.

36. Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, et al. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci. 2001;4(7):702-10.

37. Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, et al. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. The Journal of biological chemistry. 2006;281(30):21362-8.

38. Belzeaux R, Bergon A, Jeanjean V, Loriod B, Formisano-Tréziny C, Verrier L, et al. Responder and nonresponder patients exhibit different peripheral transcriptional signatures during major depressive episode. Translational psychiatry. 2012;2(11):e185.

39. Bocchio-Chiavetto L, Maffioletti E, Bettinsoli P, Giovannini C, Bignotti S, Tardito D, et al. Blood microRNA changes in depressed patients during antidepressant treatment. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2013;23(7):602-11.

40. Silva AM, Almeida MI, Teixeira JH, Ivan C, Oliveira J, Vasconcelos D, et al. Profiling the circulating miRnome reveals a temporal regulation of the bone injury response. Theranostics. 2018;8(14):3902-17.

41. Tanaka T, Shibazaki A, Ono R, Kaisho T. HSP70 mediates degradation of the p65 subunit of nuclear factor kappaB to inhibit inflammatory signaling. Science signaling. 2014;7(356):ra119.

42. Cutress RI, Townsend PA, Brimmell M, Bateman AC, Hague A, Packham G. BAG-1 expression and function in human cancer. British Journal of Cancer. 2002;87(8):834-9.

43. Southern SL, Collard TJ, Urban BC, Skeen VR, Smartt HJ, Hague A, et al. BAG-1 interacts with the p50–p50 homodimeric NF-κB complex: implications for colorectal carcinogenesis. Oncogene. 2012;31(22):2761-72.

44. Brown GC, Neher JJ. Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. Mol Neurobiol. 2010;41.

45. Chéret C, Gervais A, Lelli A, Colin C, Amar L, Ravassard P, et al. Neurotoxic activation of microglia is promoted by a nox1-dependent NADPH oxidase. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008;28(46):12039-51.

46. Tzanoulinou S, Sandi C. The Programming of the Social Brain by Stress During Childhood and Adolescence: From Rodents to Humans. Current topics in behavioral neurosciences. 2017;30:411-29.

47. Negele A, Kaufhold J, Kallenbach L, Leuzinger-Bohleber M. Childhood Trauma and Its Relation to Chronic Depression in Adulthood. Depress Res Treat. 2015;2015:650804-.

48. Heim C, Binder EB. Current research trends in early life stress and depression: review of human studies on sensitive periods, gene-environment interactions, and epigenetics. Experimental neurology. 2012;233(1):102-11.

49. Marquez C, Poirier GL, Cordero MI, Larsen MH, Groner A, Marquis J, et al. Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression. Translational psychiatry. 2013;3:e216.

50. Papilloud A, Guillot de Suduiraut I, Zanoletti O, Grosse J, Sandi C. Peripubertal stress increases play fighting at adolescence and modulates nucleus accumbens CB1 receptor expression and mitochondrial function in the amygdala. Translational psychiatry. 2018;8(1):156.

51. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nature Reviews Neuroscience. 2009;10(6):397-409.

52. Nandam LS, Brazel M, Zhou M, Jhaveri DJ. Cortisol and Major Depressive Disorder-Translating Findings From Humans to Animal Models and Back. Frontiers in psychiatry. 2020;10:974-.

53. Federenko IS, Nagamine M, Hellhammer DH, Wadhwa PD, Wüst S. The Heritability of Hypothalamus Pituitary Adrenal Axis Responses to Psychosocial Stress Is Context Dependent. The Journal of Clinical Endocrinology & Metabolism. 2004;89(12):6244-50.

54. Walker SE, Zanoletti O, Guillot de Suduiraut I, Sandi C. Constitutive differences in glucocorticoid responsiveness to stress are related to variation in aggression and anxiety-related behaviors. Psychoneuroendocrinology. 2017;84:1-10.

55. Calcia MA, Bonsall DR, Bloomfield PS, Selvaraj S, Barichello T, Howes OD. Stress and neuroinflammation: a systematic review of the effects of stress on microglia and the implications for mental illness. Psychopharmacology. 2016;233(9):1637-50.

56. Rohleder N. Stimulation of systemic low-grade inflammation by psychosocial stress. Psychosomatic medicine. 2014;76(3):181-9.

57. de Kloet ER, Karst H, Joëls M. Corticosteroid hormones in the central stress response: Quick-and-slow. Frontiers in Neuroendocrinology. 2008;29(2):268-72.

58. Wilkinson PO, Goodyer IM. Childhood adversity and allostatic overload of the hypothalamic-pituitary-adrenal axis: a vulnerability model for depressive disorders. Development and psychopathology. 2011;23(4):1017-37.

59. Johnson SA, Fournier NM, Kalynchuk LE. Effect of different doses of corticosterone on depression-like behavior and HPA axis responses to a novel stressor. Behavioural brain research. 2006;168(2):280-8.

60. Dutcher EG, Pama EAC, Lynall M-E, Khan S, Clatworthy MR, Robbins TW, et al. Early-life stress and inflammation: A systematic review of a key experimental approach in rodents. Brain and Neuroscience Advances. 2020;4:2398212820978049.

61. Tzanoulinou S, Riccio O, de Boer MW, Sandi C. Peripubertal stress-induced behavioral changes are associated with altered expression of genes involved in excitation and inhibition in the amygdala. Translational psychiatry. 2014;4:e410.

62. Salk RH, Hyde JS, Abramson LY. Gender differences in depression in representative national samples: Meta-analyses of diagnoses and symptoms. Psychological bulletin. 2017;143(8):783-822.

63. Freeman A, Mergl R, Kohls E, Székely A, Gusmao R, Arensman E, et al. A cross-national study on gender differences in suicide intent. BMC Psychiatry. 2017;17(1):234.

64. Goodwill HL, Manzano-Nieves G, Gallo M, Lee H-I, Oyerinde E, Serre T, et al. Early life stress leads to sex differences in development of depressive-like outcomes in a mouse model. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2019;44(4):711-20.

65. Tang C-Z, Zhang D-F, Yang J-T, Liu Q-H, Wang Y-R, Wang W-S. Overexpression of microRNA-301b accelerates hippocampal microglia activation and cognitive impairment in

mice with depressive-like behavior through the NF-κB signaling pathway. Cell Death & Disease. 2019;10(4):316.

66. Osimo EF, Baxter LJ, Lewis G, Jones PB, Khandaker GM. Prevalence of low-grade inflammation in depression: a systematic review and meta-analysis of CRP levels. Psychological medicine. 2019;49(12):1958-70.

67. Kim J, Yoon S, Lee S, Hong H, Ha E, Joo Y, et al. A double-hit of stress and low-grade inflammation on functional brain network mediates posttraumatic stress symptoms. Nature communications. 2020;11(1):1898.

68. Hijmans JG, Diehl KJ, Bammert TD, Kavlich PJ, Lincenberg GM, Greiner JJ, et al. Influence of Overweight and Obesity on Circulating Inflammation-Related microRNA. MicroRNA (Shariqah, United Arab Emirates). 2018;7(2):148-54.

69. Hijmans JG, Diehl KJ, Bammert TD, Kavlich PJ, Lincenberg GM, Greiner JJ, et al. Association between hypertension and circulating vascular-related microRNAs. Journal of human hypertension. 2018;32(6):440-7.

70. Wang X, Wang B, Zhao J, Liu C, Qu X, Li Y. MiR-155 is involved in major depression disorder and antidepressant treatment via targeting SIRT1. Biosci Rep. 2018;38(6):BSR20181139.

71. Su W, Aloi MS, Garden GA. MicroRNAs mediating CNS inflammation: Small regulators with powerful potential. Brain, behavior, and immunity. 2016;52:1-8.

72. Li X, Kong D, Chen H, Liu S, Hu H, Wu T, et al. miR-155 acts as an anti-inflammatory factor in atherosclerosis-associated foam cell formation by repressing calcium-regulated heat stable protein 1. Scientific Reports. 2016;6(1):21789.

73. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, et al. Chronic inflammation in the etiology of disease across the life span. Nat Med. 2019;25(12):1822-32.