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CIÊNCIAS BIOMÉDICAS

Development of functionalized nanoparticles for the stabilization and repair of central nervous system myelin under assault by acquired demyelinating diseases

Rui Pedro Moura

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2024

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D.ICBAS **2024**

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Rui Pedro Pires de Moura

Development of functionalized nanoparticles for the stabilization and repair of central nervous system myelin under assault by acquired demyelinating diseases

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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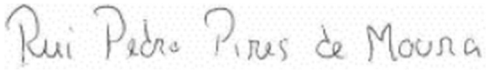

Rui Pedro Pires de Moura

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I dedicate this solely to the

memory of my best friend, my cat Anão.

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List of publications

Ao abrigo do disposto do no 2, alínea a) do artigo 31°do Decreto-Lei n.°115/2013 de 7 de agosto, fazem parte integrante desta tese de doutoramento os seguintes trabalhos já publicados ou submetidos para publicação:

All authors of the articles were aware of their use in the present thesis and did not raise any objection.

Moura, RP., Almeida, A., Sarmento, B., "The role of non-endothelial cells on the penetration of nanoparticles through the blood-brain barrier.", Progress in Neurobiology, 2017, **159**, p: 39-49. doi: 10.1016/j.pneurobio.2017.09.001.

In this review paper, I was responsible for the conception, execution and writing of the manuscript. Andreia Almeida was responsible for early proof-reading. Bruno Sarmento was responsible for supervising, revising, and submitting the paper. This paper has not been included in other thesis and is partially reproduced in the thesis Chapter 1 as authorized by the Journal Author Rights at Elsevier and Progress in Neurobiology.

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Moura, RP., Martins, C., Pinto, S., Sousa, F., Sarmento, B., "Blood-brain barrier receptors and transporters: an insight on their function and how to exploit them through nanotechnology.", Expert Opinion on Drug Delivery, 2019, **16**(3), p: 271-285. doi: 10.1080/17425247.2019.1583205.

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Moura, RP., Sarmento, B., “Therapeutic Approaches toward Multiple Sclerosis: Where Do We Stand and Where Are We Headed?”, Advanced Therapeutics, 2019. doi: doi.org/10.1002/adtp.201900070.~

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Moura, RP., Pacheco, C., Pêgo, AP., des Rieux, A., Sarmento, B., Lipid nanocapsules to enhance drug bioavailability to the central nervous system, Journal of Controlled Release, 322, 390-400, 2020.

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Faria P, Pacheco C, **Moura RP**, Sarmento B, Martins C. Multifunctional nanomedicine strategies to manage brain diseases. Drug Delivery and Translational Research, 2023, **13**(5), p:1322-1342. doi: 10.1007/s13346-022-01256-w. Epub 2022 Nov 7. PMID: 36344871.

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Moura, RP., Carvalho, ED., Martins, C., des Rieux, A., Pêgo, AP., Sarmento, B. “Functionalized retinoic acid lipid nanocapsules promotes a two-front attack on inflammation and lack of demyelination on neurodegenerative disorders”, Journal of Controlled Release, 2023, 358, p: 43-58, 10.1016/j.jconrel.2023.04.034

In this original paper, I was responsible for the conception, methodology, validation, the original research, data analysis, writing and execution. Eva Daniela Carvalho was responsible for methodology (OPCs cell culture), data curation and early proof-reading. Claúdia Martins was responsible for methodology (blood-brain barrier permeability model) and early proof-reading. Anne des Rieux and Ana Paula Pêgo were responsible for conceptualization, supervision and revising. Bruno Sarmento was responsible for funding acquisition, project administration, supervision, revising and submitting the paper. This paper has not been included in other thesis and been reproduced as is in Chapter 3 and is open access and authorized by the Creative Commons CC-BY license.

Moura, RP., Mwema, A., Bottemanne, P., Ucakar, B., Vanverenberg, K., Pêgo, A.P., Muccioli, G.G., Sarmento, B., des Rieux, A. “Retinoic acid lipid nanocapsules impact in an in vivo mice model of experimental autoimmune encephalomyelitis”, *Submitted.*

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“Always remember: Never accept the world as it appears to be. **Dare to see it for what it could be.”**

Harold Winston

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Abstract

Central nervous system disorders (CNS) have been increasing steadily as of recent years, due to an overall aging population and an increase in the diagnosis of the aforementioned pathologies. Despite there being a vast number of medical and scientific advances in recent years, therapeutics that are aimed at the CNS are still hard to come by, and there is a growing need to address it urgently to promote novel solutions. Amongst these diseases, degenerative disorders are amongst some of the most debilitating and with the highest cost burden for the health systems around the world due to the degree of incapacitation it causes in the patients. Multiple sclerosis (MS) particularly is a multifactorial, chronic inflammatory disease that is characterized by the frequent appearance of observable lesions that result from a chronic demyelinating state. Depending on the lesion site, it translates to cognitive and physical impairments that will ultimately hinder the quality of life of patients.

Intravenous drug deliver therapeutic shortcomings of the CNS usually result due to the presence of the blood-brain barrier (BBB), which is a unique endothelial layer that separates the CNS from the rest of the body, significantly limiting therapeutic effectiveness of compounds. Therefore, there is the need to address therapeutic strategies that can either circumvent or penetrate the BBB in an adequate manner in order to allow the drugs to reach bioactive concentrations at the CNS. Retinoic acid is one of such drugs, with demonstrated effectiveness in stimulating neural cell differentiation and inflammatory control but suffers from the inability to satisfactorily reach the CNS when administered in circulation, due to not being able to bypass the BBB.

Using nanotechnology to shuttle drugs across the BBB, through the use of active targeting of specific surface receptors and transporters at the endothelial cells of the BBB has been an emerging field for therapeutics of the CNS, although very little have made clinical progress. By targeting these surface proteins, the drug can reach the CNS carried in a “trojan horse” like structure, preventing the BBB from exerting its filtrating properties, and effectively penetrating it and delivering the drug inside in a satisfactory manner. The selection of proper ligands that can seamlessly interact with the BBB without many negative consequences is therefore paramount to achieve the goal. Thus, the use of these nanotechnology-based systems is a promising pathway to follow and to explore to improve the ability of drugs to reach the brain.

Building upon this knowledge and these ideas, the aim of this thesis was to develop targeted, safe and tolerable nanosystems that were able to deliver retinoic acid to the CNS and allow it to carry on its effect against MS, effectively supressing the inflammatory behaviour at the CNS and promoting remyelination, to repair the sites of demyelinating lesions. The first part of the thesis focused on rational design and validation of a complete lipid nanocapsule (LNC) based system that could encapsulate retinoic acid in satisfactory rates, associated with super paramagnetic iron oxide

nanoparticles (SPIONs), and further functionalize it with a transferrin-receptor binding peptide (TfRp) that was able to target the overexpressed transferrin receptor at the BBB to shuttle the nanosystem across.

The developed nanocapsules had ideal properties for CNS administration, presenting a slightly negative zeta potential, a monodisperse population with sizes ranging from 70-100nm depending on the external functionalization. The system was then validated for its safety, namely by assessing the cytotoxicity it exerted on cells it will interact with (BBB endothelial cells, microglia, oligodendrocyte progenitor cells), where it demonstrated a clear linear dose dependent toxicity, with a clearly defined safety range (viability levels around 90-100%) that allowed work to be carried on. Then, the specificity of the nanosystem was assessed to validate that our functionalization strategy was working and effectively promoting an increase of the accumulation of the LNC in target cells, by verifying its uptake both quantitative and qualitatively in endothelial cells of the BBB. It was found that the presence of the TfRp at the surface of the LNC increased the accumulation within these cells by 2-3-fold compared to non-modified particles and about 1.5-2-fold when saturating the transferrin receptor. To follow up on this study, a monolayer of endothelial cells was developed to validate the transport of the LNC in a model mimicking the BBB, and it resulted in a 5-fold increase of the accumulation regarding the functionalized LNC compared to the unfunctionalized LNC. After the safety and specificity were addressed, the efficacy of the system was validated to confirm that the expected biological effect of the retinoic acid was maintained when encapsulated within the system. In an LPS-induced model of microglia cells, the presence of retinoic acid reduced inflammatory biomarkers by around 50-70%. Also, the nanocapsules stimulated the differentiation of oligodendrocyte progenitor cells into myelin producing oligodendrocytes by 3-5-fold, as evaluated by imaging analysis.

Finally, the TfRp-functionalized LNCs were assessed in *in vivo* models to validate the biodistribution of the nanosystem and its ability to reach the CNS. First, tentative studies regarding its interaction with human blood to see if it activated the clotting system showed that the system was safe and tolerable and did not promote the clotting in healthy donor blood more than the positive control. Afterwards, on healthy mice, the LNC showed the ability to accumulate in acceptable rates at the CNS of healthy mice. When comparing the routes of administration, bypassing the BBB through the intranasal routes, and directly targeting it through intravenous route showed that the intravenous route resulted in 1.5-fold more accumulation within the CNS. However, this difference did not seem to result from the process functionalization, as it was not satisfactorily increasing the accumulation of the LNC within the brain of the mice compared to the non-functionalized LNC, and it seemed to promote generalized toxicity in defined biomarkers and following the weight of the animals. To finalize, the LNC were administered in an MS-replicating model, experimental autoimmune encephalomyelitis, where the LNC clearly showed the ability to improve the clinical scoring of affected mice and was able to retard

the disease onset and the maximum clinical scoring of the mice, while also being able to reduce the unstained portion of fluoromyelin, suggesting it could preserve some myelin function. The LNC in itself seemed to show a moderate effect suggesting that the vehicle in itself had a role *in vivo.*

Overall, a BBB-targeted LNC nanosystem was developed to deliver retinoic acid to increase its accumulation in the CNS. The LNC showed to have ideal properties for CNS delivery, to be safe and tolerable for administration in both acute and subacute exposure, to result in enhanced accumulation in endothelial cells of the BBB and demonstrated clear efficacy in acting in the two pillars of MS, inflammation, and demyelination. However, when translated towards an *in vivo* model, the functionalized LNC seemed to have some unexpected toxicity effects that warrant some consideration and did not sufficiently improve the CNS accumulation of the LNC compared to the unfunctionalized version. The anti-inflammatory effect noticed in cellular models did not seem to be translatable towards *t*he *in vivo* model, with the LNC only achieving a very modest reduction of pro-inflammatory biomarkers, and a slight increase towards IFNγ production. Despite this, the LNC were able to reduce the unstained area of fluoromyelin in spinal cord sections of mice, suggesting that they are able to preserve myelin function in an ongoing demyelinating disorder.

Resumo

As doenças do sistema nervoso central (SNC) têm vindo a aumentar de forma constante nos últimos anos, devido ao envelhecimento global da população e ao aumento do diagnóstico das patologias referidas. Apesar dos inúmeros avanços médicos e científicos registados nos últimos anos, as terapêuticas dirigidas ao SNC continuam a ser difíceis de encontrar, havendo uma necessidade crescente de as abordar com urgência para promover novas soluções. Entre estas doenças, as doenças degenerativas são identificadas entre as mais debilitantes e com os custos mais elevados para os sistemas de saúde de todo o mundo, devido ao grau de incapacidade que provocam nos doentes. A esclerose múltipla (EM), em particular, é uma doença inflamatória crónica multifatorial que se caracteriza pelo aparecimento frequente de lesões observáveis que resultam de um estado desmielinizante crónico. Dependendo do local da lesão, esta traduz-se em deficiências cognitivas e físicas que, em última análise, prejudicam a qualidade de vida dos doentes.

As lacunas terapêuticas da administração intravenosa de medicamentos no SNC resultam geralmente da presença da barreira hemato-encefálica (BHE), que é uma barreira endotelial única que separa o SNC do resto do corpo, limitando significativamente a eficácia terapêutica dos compostos. Por conseguinte, é necessário abordar estratégias terapêuticas que possam contornar ou penetrar na BHE de forma adequada, a fim de permitir que os fármacos atinjam concentrações bioactivas no SNC

O ácido retinóico é um desses fármacos, com eficácia demonstrada na estimulação da diferenciação das células neurais e no controlo da inflamação, mas sofre da incapacidade de atingir satisfatoriamente o SNC quando administrado em circulação, por não conseguir contornar a BHE.

A utilização da nanotecnologia para transportar fármacos através da BHE, através da utilização de receptores e transportadores de superfície específicos nas células endoteliais da BHE, tem sido um campo emergente para a terapêutica do SNC, embora muito poucos tenham feito progressos clínicos. Ao visar estas proteínas de superfície, o fármaco pode chegar ao SNC transportado numa estrutura semelhante a um "cavalo de Troia", impedindo a BHE de exercer as suas propriedades filtrantes, penetrando eficazmente na mesma e libertando o fármaco no seu interior de forma satisfatória. A seleção de ligandos adequados que possam interagir perfeitamente com a BHE sem muitas consequências negativas é, portanto, fundamental para atingir o objetivo. Assim, a utilização destes sistemas baseados na nanotecnologia é um caminho

promissor a seguir e a explorar para melhorar a capacidade dos fármacos de chegarem ao cérebro.

Com base neste conhecimento e nestas ideias, o objetivo desta tese foi desenvolver nanosistemas direccionados, seguros e toleráveis, capazes de transportar o ácido retinóico para o SNC e permitir que este exerça o seu efeito contra a EM, suprimindo eficazmente o comportamento inflamatório no SNC e promovendo a remielinização, para reparar os locais das lesões desmielinizantes. A primeira parte da tese centrou-se na conceção racional e validação de um sistema completo baseado em nanocápsulas lipídicas (LNC) capaz de encapsular ácido retinóico em quantidades satisfatórias, associado a nanopartículas de óxido de ferro super paramagnético (SPIONs), e ainda funcionalizá-lo com um péptido de ligação ao recetor da transferrina (TfRp) capaz de reconhecer o recetor da transferrina sobre-expresso na BBB para transportar o nanosistema.

As LNC desenvolvidas tinham propriedades ideais para administração no SNC, apresentando um potencial zeta ligeiramente negativo, uma população monodispersa com tamanhos que variavam entre 70-100nm, dependendo da funcionalização externa. O sistema foi então validado quanto à sua segurança, nomeadamente através da avaliação da citotoxicidade que exerce sobre as células com as quais vai interagir (células endoteliais da BHE, microglia, células progenitoras de oligodendrócitos), onde demonstrou uma clara toxicidade linear dose-dependente, com um intervalo de segurança claramente definido (níveis de viabilidade na ordem dos 90-100%) que permitiu a continuação do trabalho. Em seguida, a especificidade do nanosistema foi avaliada para validar que a nossa estratégia de funcionalização estava a funcionar e a promover efetivamente um aumento da acumulação das LNC nas células alvo, verificando a sua absorção quantitativa e qualitativa nas células endoteliais da BHE. Verificou-se que a presença do TfRp na superfície das LNC aumentou a acumulação nestas células em 2-3 vezes em comparação com partículas não modificadas e cerca de 1,5-2 vezes ao saturar o recetor de transferrina. Para dar seguimento a este estudo, foi desenvolvida uma monocamada de células endoteliais para validar o transporte das LNC num modelo que imita a BHE, o que resultou num aumento de 5 vezes da acumulação relativamente ás LNC funcionalizadas em comparação com as LNC não funcionalizadas. Após a segurança e a especificidade terem sido abordadas, a eficácia do sistema foi validada para confirmar que o efeito biológico esperado do ácido retinóico foi mantido quando encapsulado no sistema. Num modelo de células da microglia induzido por LPS, a presença de ácido retinóico reduziu os biomarcadores inflamatórios em cerca de 50-70%. Além disso, as LNC estimularam a diferenciação de células progenitoras de

oligodendrócitos em oligodendrócitos produtores de mielina em 3-5 vezes, conforme avaliado por análise de imagem.

Finalmente, as LNCs funcionalizadas com TfRp foram avaliados em modelos in vivo para validar a biodistribuição do nanosistema e sua capacidade de atingir o SNC. Em primeiro lugar, estudos preliminares relativos à sua interação com o sangue humano para verificar se activava o sistema de coagulação mostraram que o sistema era seguro e tolerável e não promovia a coagulação no sangue de dadores saudáveis mais do que o controlo positivo.

Posteriormente, em ratos saudáveis, as LNC mostrou a capacidade de se acumular em taxas aceitáveis no SNC de ratos saudáveis. Quando se comparam as vias de administração, contornando a BBB através das vias intranasais e visando-a diretamente através da via intravenosa, verificou-se que a via intravenosa resultou numa acumulação 1,5 vezes maior no SNC. No entanto, esta diferença não pareceu resultar da funcionalização do processo, uma vez que não aumentou satisfatoriamente a acumulação das LNC no cérebro dos ratinhos, e pareceu promover uma toxicidade generalizada em biomarcadores definidos e no seguimento do peso dos animais. Para finalizar, as LNC foram administradas num modelo que replica a EM, a encefalomielite autoimune experimental, onde as LNC mostraram claramente a capacidade de melhorar a pontuação clínica dos ratos afectados e foi capaz de retardar o início da doença e a pontuação clínica máxima dos ratos, ao mesmo tempo que foi capaz de reduzir a porção não corada da fluoromielina, sugerindo que poderia preservar alguma função da mielina. As LNC, por si só, pareceram mostrar um efeito moderado, sugerindo que o veículo, por si só, tinha um papel *in vivo*.

De um modo geral, foi desenvolvido um nanosistema LNC direcionado para a BHE para administrar ácido retinóico e aumentar a sua acumulação no SNC. As LNC demonstraram ter propriedades ideais para a administração no SNC, serem seguras e toleráveis para administração , e após exposição aguda e subaguda, resultaram numa maior acumulação nas células endoteliais da BHE e demonstraram uma eficácia clara nos dois pilares da EM, inflamação e desmielinização. No entanto, quando administradas em um modelo *in vivo*, as LNC funcionalizadas pareceram ter alguns efeitos de toxicidade inesperados que merecem alguma consideração e não melhorou suficientemente a acumulação das LNC no SNC em comparação com a versão não funcionalizada. O efeito anti-inflamatório observado nos modelos celulares não parece ser traduzível para o modelo *in vivo*, com as LNC a conseguir apenas uma redução muito modesta dos biomarcadores pró-inflamatórios e um ligeiro aumento da produção de IFNγ. Apesar disso, as LNC foram capazes de reduzir a área não corada de fluoromielina em secções da

medula espinhal de ratos, sugerindo que são capazes de preservar a função da mielina numa doença desmielinizante em curso.

I have self-doubt. I have insecurity.

I have a fear of failure.

I have nights when I show up at the arena and I'm like,

'My back hurts, my feet hurt, my knees hurt. I don't have it. I just want to chill.'

We all have self-doubt.

You don't deny it, but you also don't capitulate to it.

You embrace it.

Kobe Bryant

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Acronyms and Abbreviations

Ajs – Adherens junctions

ALT – Alanine transferase

ANOVA – Analysis of variance

ATF3 – Activating transcription factor 3

Papp – Apparent permeability

AST – Aspartate transferase

AE – Association efficiency

ATF3 – Activating transcription factor 3

AUC – Area under the curve

bFGF – Basic fibroblast growth factor

BBB – Blood-brain barrier

BUN – Blood urea/nitrogen

CNS – Central nervous system

CFA – Complete freund adjuvant

DiD – 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt

DiR – 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindotricarbocyanine iodide

DMEM – Dulbecco’s modified Eagle medium

DMSO – Dimethyl sulfoxide

DMT – Disease modifying therapy

ESI – Electrospray ionization

EBM-2 – Endothelial basal medium-2

ELISA – Enzyme linked immunosorbent assay

EDTA – Ethylenediaminetetraacetic acid

EAE – Experimental autoimmune encephalomyelitis

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

FLI – Fluorescence imaging

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HBSS – Hank's balanced salt solution

H&E – Hematoxilin and eosin

HPLC – High performance liquid chromatography

IL-6 – Interleukin-6

IL-10 – Interleukin-10

IL-12 – Interleukin-12

IL-23 Interleukin-23

LNC-DiD – DiD-loaded lipid nanocapsules

LNC RA-DiR – DiR-loaded lipid nanocapsules

LNC – Lipid nanocapsules

LPS – Lipopolysaccharide

LNC RA – Retinoic acid-loaded lipid nanocapsules

MOG – Myelin oligodendrocyte glycoprotein

mRNA – Messenger RNA

MYRF – Myelin gene regulating factor

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MFU – Media fluorescence intensity

MS – Multiple sclerosis

MBP – Myelin basic protein

NALT – Nasopharinx-associated lymphoid tissue

NSCs – Neural stem cells

NO – Nitric oxide

NDS – Normal donkey serum

ND – Not detected

NMR – Nuclear magnetic resonance

OPCs – Oligodendrocyte progenitor cells

OCT – Optimal cutting temperature compound

PCR – Polymerase chain Reaction

PFA – Paraformaldehyde

PTX – Pertussis toxin

PBS – Phosphate buffered saline

PLL – Poly-L-lysine

PPMS -Primary progressive Multiple Sclerosis

PRMS – Progressive relapsing Multiple Sclerosis

PdI – Polydispersity Index

PLGA – Poly(lactic-co-glycolic) acid

qPCR – quick polymerase chain reaction

RRMS – Relapsing remitting multiple sclerosis

RA – Retinoic acid

RAR – Retinoid-A-receptor

RARα – Retinoid-A-receptor α

RARβ – Retinoid-A-receptor β

RXR – Retinoid-X-receptor

RNA – Ribonucleic acid

RPL19 – Ribosomal protein L19

RT – Room temperature

SEM – Scanning electron microscopy

SPMS – Secondary progressive multiple sclerosis

SIM – Single ion monitoring

NaCl – Sodium chloride

SD – Standard deviation

SPIONs – Super paramagnetic iron oxide nanoparticles

Th – T helper cell

TJs – Tight junctions

TfR – Transferrin receptor

TfRp – Transferrin receptor binding peptide

TfRp-LNC – Transferrin receptor binding peptide functionalized lipid nanocapsules

TfRp-SPION-LNC DiD – Transferrin receptor binding peptide functionalized DiD-super paramagnetic iron oxide nanoparticles-loaded lipid nanocapsules

TfRp-LNC RA-DiR – Transferrin receptor binding peptide functionalized retinoic acid-DiR-loaded lipid nanocapsules

TfRp-LNC RA – Transferrin receptor binding peptide functionalized retinoic acid-loaded lipid nanocapsules

TfRp-SPION-LNC RA – Transferrin receptor binding peptide functionalized retinoic acid-super paramagnetic iron oxide nanoparticles-loaded lipid nanocapsules

TEM – Transmission electron microscopy

TEER – Trans endothelial electrical resistance

TNF-α – Tumor necrosis factor alpha

USA – United States of America

v/v – volume/volume solution

WHO – World Health Organization

CHAPTER 1 – LITERATURE REVIEW

This chapter was adapted from published reviews and book chapters:

Moura, RP., Sarmento, B., “Therapeutic Approaches toward Multiple Sclerosis: Where Do We Stand and Where Are We Headed?”, Advanced Therapeutics, 2019. Doi: doi.org/10.1002/adtp.201900070.

Moura, RP., Martins, C., Pinto, S., Sousa, F., Sarmento, B., “Blood-brain barrier receptors and transporters: an insight on their function and how to exploit them through nanotechnology.”, Expert Opinion on Drug Delivery, 2019, 16(3), p: 271-285. Doi: 10.1080/17425247.2019.1583205.

Moura, RP., Sousa, F., Almeida, A., Pinto, S., Sarmento, B., “Theranostic biomaterials for the regulation of the blood-brain barrier” in Theranostic Biomaterials, 2019. Doi: 10.1016/B978-0-12-815341-3.00013-4.

Moura, RP., Almeida, A., Sarmento, B., “The role of non-endothelial cells on the penetration of nanoparticles through the blood-brain barrier.”, Progress in Neurobiology, 2017, 159, p: 39-49. Doi: 10.1016/j.pneurobio.2017.09.001.

1- Multiple sclerosis

Diseases relating to the central nervous system (CNS), ranging from neurodegeneration to infections, cancers or traumatic events affect approximately 300 million people worldwide, with a global cost of 790 billion (€) per year, as reported by an estimate from 2010 [1]. Furthermore, CNS disorders are responsible for 16-17% of worldwide deaths, second only to cardiovascular diseases [2]. Thus, CNS disorders are some of the most devastating conditions not only in lethality, but also regarding the quality of life. Harsh symptoms, aggressive behavior and lack of therapeutic options make these pathologies some of the most feared group of conditions. With the increase in the standards of healthcare, the median life expectancy also increases leading to a higher number of people with 65 years of age or more [3]. Indeed, a study conducted in 2015 by the United Nations points to the number of people aged 65 or higher to double by 2050 [4]. Individuals of this age group are more prone to neurological disorders, primarily neurodegeneration such as Alzheimer's, Parkinson's, or MS [5]. Therefore, it is foreseen that through an increase in the elderly population, neurological disorders will continue to increase in both incidence and prevalence in the world, requiring a clinical focus on them to increase therapeutic options. Due to this, there is an innate need to start researching therapeutic options for this type of diseases.

Multiple sclerosis (MS) specifically is a chronic, debilitating, progressive degeneration of the myelin sheath that surrounds axons resulting in impaired signal transduction between neurons. Although it is not considered a fatal disease in our times, it significantly impairs the quality of life of an individual, primarily regarding their locomotion and ability to carry on regular every-day activities, effectively impending afflicted patients' from maintain an independent life. It affects an estimated 2.9 million people worldwide [6] and can affect people of all ages. It tends to affect primarily women, compared to men (2.5:1 reported ratio) [7], while diagnosis is usually on adulthood (between 20 and 50 years of age), with a median age of onset of around 39 years. It is the most common neurodegeneration amongst young adults and has a higher prevalence in developed countries and in countries further from the equator with less solar exposure [8].

1.1- Pathophysiology and pathogenesis of multiple sclerosis

First and foremost, MS is characterized by a general, chronically progressive state of inflammation occurring at the CNS [9]. It can be considered a multifactorial disease as it can be traced to genetic predispositions, exposure to certain infectious agents or environmental factors [10] It is commonly theorized that the interactions between these factors are required to trigger the beginning of the disease. The main features that are observable within multiple sclerosis is clear and evident lesions or zones of demyelination due to oligodendrocyte death observable by magnetic resonance imaging (MRI) as well as tracking attacks of neurological dysfunction within the last 24-48 hours [11].

Certain genetic predispositions can increase the risk of development of MS, but not directly lead to its appearance. One of the most notorious and confirmed examples is in the human leukocyte antigen (HLA) region of chromosome six, a gene mainly responsible for encoding for the major histocompatibility complex (MHC) proteins. Alterations in this gene complex can result in both susceptible and protective effects regarding MS [7, 12, 13].

1.2– The immune system dysregulation in MS

Although plenty of theories are present regarding MS, it is well accepted that it stems from an autoimmune origin (Figure 1). MS is primarily characterized by defined inflammatory infiltrates at the CNS that leads to the formation of MRI-detectable demyelinating plaques. These infiltrates are typically made up of T-lymphocytes, although B-cells and macrophages are also detectable in lower quantities [14]. A pivotal checkpoint to begin the downstream inflammatory cascade is the interaction between T-CD4+ cells with microglia (acting as an antigen presenter cell). This in turns promotes production of pro-inflammatory interleukins, namely IL-12 and IL-23 that will promote T-cell differentiation into an inflammatory phenotype, such as Th1 and Th17 [15].

The inflammatory process of the central nervous system, affecting both white and grey matter alike, is closely related to an aberrant reaction of both adaptive and innate immune response. It occurs due to the penetration of rogue immune cells, and the production of cytokines which will ultimately target and damage the myelin sheaths of neurons. As immune cells hold the ability to freely cross the blood-brain barrier (BBB), through the interaction between their integrins with cell adhesion molecules, little resistance is offered on this step [16]. A possible

factor regarding the beginning of leukocyte infiltration can be related to chemokine signaling, although nothing confirmatory as yet been found [17]. Studies point towards the interaction of T CD4+ (T-Helper) cells with antigen presenting cells (APCs), such as dendritic cells or microglia, as a pivotal role in the triggering and progression of the disease [18]. The interaction between the pathogen and APCs will trigger the production of certain interleukins (IL-12, IL-23) by these cells that will trigger T CD4+ differentiation into varying phenotypes, namely Th1 and Th17 [7]. The Th1 phenotype has the ability to produce both proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) or interferon gamma (IFNγ) [19]. Not only this, but these cytokines can transiently suppress the differentiation of T CD4+ into Th2, a phenotype capable of producing anti-inflammatory cytokines and suppressing an impending immune response [20]. Th17 also produces proinflammatory cytokines such as IL-17, IL-21, amongst others [21]. To add to this, Th17 cells have been linked to the ability to damage the BBB, which in turn leads to a great influx of immune cells or unwanted substances towards the CNS [22].

T CD8+ (T-Cytotoxic) cells also play a role in the pathogenesis of MS, especially through their cytolytic action against glial cells or mature oligodendrocytes capable of producing myelin [23]. Furthermore, T CD8+ can trigger an apoptotic process in mature oligodendrocytes through the release of pro-apoptotic factors, such as the Fas ligand. This triggers premature apoptosis of the oligodendrocyte, resulting in lower numbers of myelin-producing capable cells [24]. In addition, T-lymphocytes can also transiently activate B-lymphocytes against myelin sheaths, usually towards myelin basic protein (MBP) or myelin-oligodendrocyte glycoprotein, resulting in the production of auto-antibodies that target and bind against these proteins to destroy myelin sheaths. Although it is uncertain why MBP is recognized as foreign, research points towards molecular mimicry of certain pathogens, such as herpesvirus-6 [25], that leads to a cross-reaction response by T-lymphocytes. The combination of these events usually results in axonal degeneration of the neurons. T CD8+ can also be activated by contacting with activated microglia, who can act as antigen presenting cells (APC) through the MHC complexes I and II, triggering the proliferation of these cells and an overall pro-inflammatory cascade [26].

In response to this, resident microglia switch to an activated phenotype, which is similar to T-lymphocytes can offer a protective role or further entice the ongoing neurodegeneration [27]. The damage that microglia can inflict on the CNS is mainly attributed to the release of toxic molecules that result from their action, such as proteases or reactive oxygen/nitrogen species [28]. Microglia represents around 12-16% [29] of the cells in the CNS of humans and can act

both as a pro-inflammatory and anti-inflammatory mediator at the CNS, depending on the external factors acted upon them [30]. Certain phenotypes, such as the CD11c+ are directly involved in the remyelination process in injury sites of MS [31]. Microglia in itself are involved in the exacerbation of MS since they can be found alongside the lesions, where they contribute towards the production of pro-inflammatory cytokines and reactive oxygen species, that trigger the apoptotic process of oligodendrocytes [32].

Thus, the whole of the immune system appears to hold a role regarding both the appearance and outcome of MS, through a plethora of different mechanisms.

1.3 – The aggravating factors in multiple sclerosis

As a multifactorial disease, environmental factors are also present in most situations where MS develops (Figure 1.1). Lifestyle factors always play a particular role, as poor habits will lead to a globally poor body homeostasis, such as cigarette smoking, lack of physical activity and uncontrolled body weight [33]. Certain factors have been successfully linked with the disease, through several cohort studies. One of the most widely presented, and accepted, is related to vitamin D. Vitamin D belongs to a group of fat-soluble molecules, with a several different effects on the human body, mainly in the regulation of calcium absorption and their role regarding the bones [34]. However, it has recently been linked to immunomodulatory effects [35], and regulation of musculoskeletal health which can explain its link to MS [36]. Its immunomodulatory effects stem mostly from the ability to significantly hinder the proliferation of Th1 lymphocytes and their respective cytokines (pro-inflammatory mediators), whilst up-regulating the expression of both Th2 and Treg lymphocytes, both who act in a pro-resolutive way regarding inflammation [37]. Not only this, but vitamin D has also been shown to stimulate the differentiation of immature oligodendrocytes in mature, capable of producing myelin, oligodendrocytes, whilst also preventing the premature apoptosis of capable oligodendrocytes [38]. These effects are corroborated when compared against epidemiological data, wherein prospective studies demonstrated that serum levels of vitamin D were lower in patients with confirmed, diagnosed MS compared to healthy individuals [39]. Furthermore, it has been found that higher circulating levels of vitamin D are linked to a lesser risk of developing MS [40]. Thus, lower levels of vitamin D are expected in patients diagnosed with MS, mainly those afflicted by

motor impairment, consistent relapses, or a progressive phenotype of MS, due to reduced sunlight exposure, the main catalyst of vitamin D production.

Recently, many studies comparing the gut-brain axis and the impact that the gut microbiota might have on neurodegeneration have been conducted, showing a link between the appearance and accumulation of toxic compounds produced by microglia in MS patients an CNS cell apoptosis [41]. Although the mechanisms behind this are not yet fully elucidated, it might be related to an alteration in the proportions of neurotransmitters, which in turn could result in altered immune responses both in the periphery and in the CNS [42].

Other commonly linked factor to MS are infectious agents. Although certain infectious agents are neurotropic and can reside in the CNS, their location should be matched to their action. Thus, the most linked agents to MS are virus, mainly from the Herpesviridae family. As stated before, herpesvirus-6 shares a homologous sequence with MBP, which can trigger cross-reactions by the immune system after it is recognized [43]. Recently, there were studies published that directly correlated Epstein-Barr virus (EBV)[30], a virus that is commonly linked to mononucleosis or asymptomatic infections in children and teens, with the appearance and the development of MS. The role of EBV has been adequately demonstrated in several autoimmune diseases, and its seropositivity has been demonstrated in close to 100% of patients afflicted by MS [44, 45]. Furthermore, another study has described the response by B-lymphocytes in MS during active EBV infection, compared to little-to-no response in other neurological conditions [46], strengthening the case for a link between a dysregulated EBV CNS infection and MS. EBV presence can also contribute to significantly lower quality of life and a bigger impact of MS progression [47].

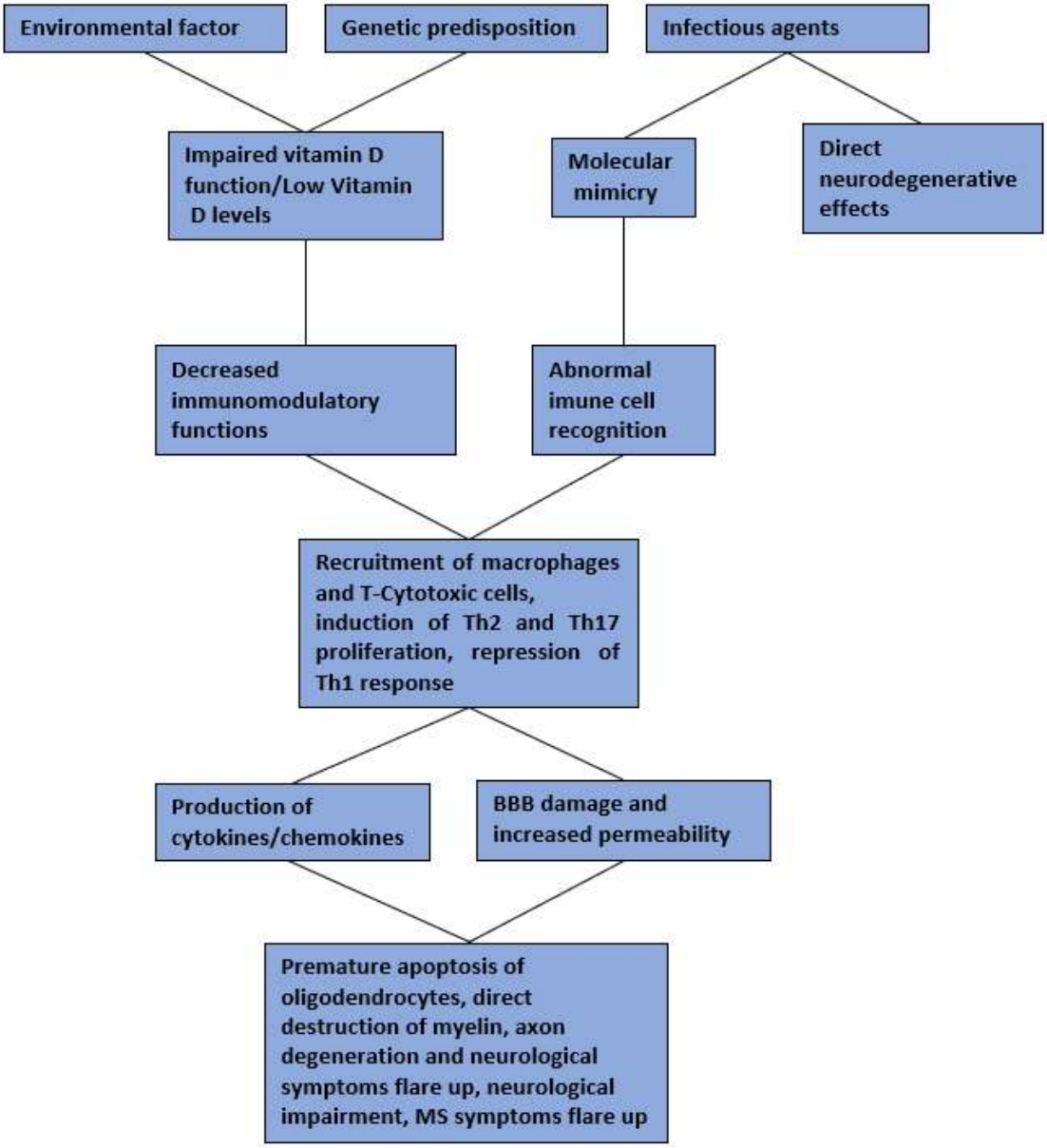

Figure 1.1. The pathogenesis pathway of multiple sclerosis demonstrating how each factor can link with each other to exacerbate the condition. Reproduced with permission from [48].

1.4 – Genetic factors

Although MS is not necessarily described as a genetic disease, it is known that descendants of MS patients are more likely to have the disease than those who don't. This is particularly significant in the case of twins, where the likelihood of one developing MS if the other one has it is about 1 in 4, compared to 1 in 1000 for the general populace [49]. Most genetic risks identified come from mutations in the genes that are responsible for the production of key players in pathogenesis of MS, such as MHC complex, or single nucleotide polymorphisms (SNPs) in innate immune response genes [49]. Generally, a combination of these and the environmental factors listed above will lead to the emergence of the disease.

1.5 – Remyelination, and how multiple sclerosis inhibits this process

Despite the looming threat of demyelination (Figure 1.2) once a neurodegeneration process begins, the body has the ability to replenish the damaged myelin through a process known as remyelination [50]. Through the mobilization of progenitor cells, named oligodendrocyte progenitor cells (OPCs), these can undergo rapid differentiation into mature oligodendrocytes and replenish the damaged myelin where required [49]. This is a process that is active day to day in the human body, although with the passage of time its efficiency wanes [51]. Despite the OPCs being the main players in the remyelination process, it should be noted that astrocytes and microglia can play a key factor in ensuring that this occurs without any issues [29].

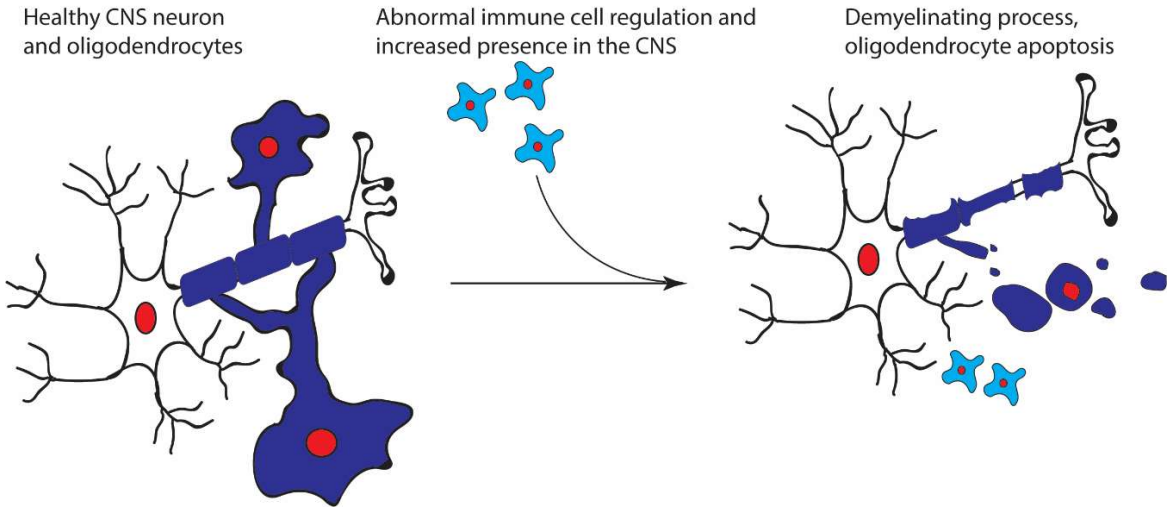

Figure 1.2. The contributing factors to demyelination and the degeneration of the myelin sheath of neurons. Reproduced and modified with permission from [48].

The process of remyelination consists of two main steps, being the activation of the OPCs, and the mobilization towards lesion sites with subsequent differentiation. The remyelination process can however be disrupted in either of these steps [52]. The activation consists in the detection of myelin debris by astrocytes and microglia, that then secrete certain factors that signal the OPCs and trigger their transcription factors that will contribute to the latter steps of mobilization and differentiation [53]. After this activation, the same astrocytes and microglia will secrete chemoattractants that will mobilize the OPCs towards the lesion location, stimulating their replication and differentiation [29]. Then, with the right differentiation factors that are secreted by the surrounding cells in the lesion locations, such as PDGF, FGF, Activin A, myelin gene regulating factor (myrf), leukemia inhibitory factor and other interleukins [54-56], the OPCs will undergo the differentiation towards oligodendrocytes, and begin producing myelin capable of sheathing the neurons around it, preventing focal demyelinating zones from arising and expanding.

In MS however, this process is significantly impaired, and the rate of remyelination occurring at the CNS is less than the rate of demyelination, eventually not occurring at all when enough damage has occurred [51]. Animal studies have shown that whatever oligodendrocytes that are mobilized to lesion sites in MS are not newer OPCs but rather older oligodendrocytes, due to expression of potential chemorepellents like semaphoring 3A or netrin-1 [57], suggesting

that there is a lack of ability of mobilization of OPCs in chronic states of MS. In humans however it has been hypothesized that not only does this process occur, but also newer OPCs can be mobilized to the lesion site [58]. Due to this, rather than pinpointing a specific action that contributes towards the failure of remyelination in MS patients, it seems that a “dysregulation hypothesis” is the most likely scenario, wherein there is a breakdown in the signaling pathways listed above, due to the changes to the environment at the CNS [51]. Due to this, a lot of research has revolved around identifying inhibitory signaling pathways in MS that might contribute towards this suppression, or pathways that favor differentiation.

Amongst the inhibitory pathways, the ones that have been studied the most are the Notch pathway or the Wnt pathway [59, 60]. Within the Notch pathway, Lingo-1 has been the one that has been explored the most with therapeutic candidates that have made it towards clinical trials, although none has succeeded in achieving a positive therapeutic outcome [61]. Regarding the differentiation pathways, there has been a special interest towards drugs that can stimulate the retinoid receptor family (retinoid X receptor and retinoid A receptor) [62].

2 – Diagnosing and understanding the clinical presentation of multiple sclerosis

Clinical MS can be presented in four different forms (Table 1.1) that differ by symptom progression and rate of relapse. It should be noted that there can be an asymptomatic variant of MS, also known as subclinical, in which the patient experiences no symptoms. It is only discovered by chance or during an autopsy [63]. The differing types of clinical MS and specific features are described in Table 1. Although the diagnostic procedures are globally the same, specific differences are applied to correctly assess the type of presentation the individual has.

Table 1.1 Differing phenotypes of MS, their frequency and clinically observed features.

Type of MS presentation	**Disease progression**	**References**
Relapsing/Remitting MS (RRMS)	Most common form of MS. Occurs in 80-85% of the cases. Usually presents itself with isolated cases of symptom exacerbation (relapse) followed by periods of symptom improvement no symptom presentation (remission) with. Most cases will eventually progress to SPMS within 20 years.	[64, 65]
Primary Progressive MS (PPMS)	Second most common form, occurring in 10% of MS cases. Characterized by a continual and progressive increase of the severity of the symptoms, without periods of remission, only occasional periods of stagnation in progress. Usually demonstrates higher degrees of resistance to medication compared to other forms.	[65, 66]
Secondary Progressive MS (SPMS)	Termed secondary as it develops usually from patients with RRMS. DMT can delay the progression towards this state. Characterized by a lack of remission periods like in PPMS, with an increase in the severity of symptoms. Usually associated by a steady neurological deterioration without the presentation of acute attacks	[9, 65]
Progressive Relapsing MS (PRMS)	The most uncommon form of MS presentation, occurring in less than 5% of the patients. Characterized by a progressive decline in cognitive and neurological functions, further exacerbated by acute attacks of increasing severity regarding the symptoms. Can only be	[9, 68]

distinguished from normal progressive states when an exacerbation occurs.	

A vast majority of the patients will initially experience a sudden episode of neurological impairment (optic neuritis, incomplete transverse myelitis or injuries at the brainstem leading to motor issues) in what is known as a first demyelinating event, that usually progresses over a few days up to weeks [69]. This clinically isolated event will eventually subside, beginning a period where no symptoms are experienced. If a different portion of the CNS is afflicted after some time, the individual has been termed to have relapsed, and the individual after confirmatory exams is diagnosed with clinically definite MS [70]. If, however, this clinically isolated event does not appear to improve on its own and progressively worsens the likely scenario is PPMS. Afterwards, the diagnostic procedure relies upon a clinical evaluation by the physician that is supported by both laboratory findings and magnetic resonance imaging (MRI) [71]. The main criteria for an adequate MS diagnosis rely upon objective evidence of two separate CNS lesions that can be comparable to MS that are separated both in time and space, compatible with two isolated attacks. This should be corroborated with a differential diagnosis of other possible causes of CNS lesions [70]. Tests to confirm involve an immunologic profile of the cerebrospinal fluid (CSF) and evoked potential electrophysiologic studies. Typically, the CSF can demonstrate oligoclonal bands, but this is not exclusive to MS. Biomarkers of neurodegeneration or CNS atrophy can also be investigated, such as neurofilament light and heavy chain (NFL and NFH respectively) which represent axon damage, glial fibrillary acidic protein which is usually a marker of abnormal glial cell activation or even quantification of MBP [72], which is increased in the CSF of most MS patients. Serologies towards certain viruses can also be conducted, as well as a serum quantification of vitamin D3. Biomarkers relating to BBB disruption have also been exploited, such as the presence of matrix metalloproteinases or gliotoxin [73, 74]. Other serum biomarkers mainly relating to immune cell activation and abnormal function have been investigated, such as osteopontin which reflects T-cell activation, soluble CD 163, which represents macrophage activation, or other chemokine/cytokine receptors, but none has been correctly assessed as clinically relevant yet [75-77]. The clinical considerations and diagnostic considerations in order to get a definite MS diagnosis follow the most updated McDonald criteria [78, 79].

Typically, the main areas affected of the CNS are the optic nerve, the spinal cord, the brainstem, and the cerebellum [80]. Sensory disturbance, such as numbness or tingling, difficulty moving that can lead to an ataxic gait, vision deficits, emotional and cognitive difficulties are the primary symptoms that results from the injuries, with secondary symptoms commonly resulting from the consequences of these [7]. This will be dependent on where the lesions are located and the extent of the neurodegeneration that has occurred. Typically, these patients will also have a damaged BBB, the main “bodyguard” of the CNS, resulting in a more vulnerable CNS than a healthy individual [81].

2.1 - Treatment approaches towards multiple sclerosis

2.1.1 Currently available treatments

As MS can present itself in different forms, with each holding varying periods of disease flare-up and periods of little to no impact on the quality of life, treatment options are discussed frequently with the clinic that accompanies the patient. During flare-ups certain medications can be used to promote better tolerance of the symptoms, to try to preserve some quality of life. A wide array of medications can be used in these situations, such as motility promoters when the gastrointestinal tract is affected, anticholinergic medication for bladder affections, and medications used to treat other neurological ailments [82]. However, this reviews’ focus in on current available primary treatment for MS and upcoming alternatives currently in trials or being developed.

Currently, MS still has no effective cure. Approved treatment options still cannot reverse the demyelinating process ongoing at the CNS, or fully halt it, instead relying upon preserving the CNS by slowing down the demyelination as much as possible. Disease modifying therapies (DMT) or immunomodulatory agents are the main source of combat against MS, and they are moderately effective at controlling or vastly slowing down the demyelinating process, mostly due to modulating and reducing the exacerbation and activation of the immune system, or a purely anti-inflammatory effect on the CNS [9]. If MS first presents with a clinically isolated syndrome that has a favorable prognosis or cases of RRMS with no relapses or positive feedback from MRI scans, patients are not advised to being therapy with a DMT. However,

DMT should be started if the clinically isolated syndrome has a poor prognosis and the patient has suffered from one or more cases of a relapse, or a worsening of the lesions seen through MRI [83]. The most common first line of DMT for MS are interferon beta, glatiramer acetate, teriflunomide and dimethyl fumarate [84, 85].

Interferon beta-1a is indicated in the presence of a clinically isolated syndrome with poor prognosis, at a dosage of 250mcg subcutaneously (sc.) every other day. It is also the primary treatment option in RRMS and SPMS. Interferon beta-1b holds the same clinical applications but is administered as 30mcg intramuscular (i.m.) once a week [85]. Interferons hold their clinical action mainly through an anti-inflammatory response through a decrease in the proliferation of T-cells, a decrease in the amount of pro-inflammatory cytokines produced by these cells, and transiently preventing their migration to the CNS [86]. The main features interferon promotes is a reduction in relapses (30-34%) and an overall improvement on the results from gadolinium-MRI, per results from a phase III trial during a 2-year follow-up of the patients [87]. However, treatment with interferon beta may induce the formation of targeted neutralizing antibodies, that can greatly reduce treatment efficiency with interferon, eventually leading to the introduction of a second-line agent [88].

Glatiramer acetate is a mix of synthetic peptides that aim to mimic MBP. The mechanism of action is not fully elucidated as of yet, but the main hypothesis seems to derive from its core structure resembling MBP, making it thus that the autoimmune response experienced is directed towards it instead of the MBP expressed by oligodendrocytes, halting their destruction. Another proposed mechanism is an activation of the transiently suppressed anti-inflammatory Th2 cells, whilst suppressing Th1 cells [89]. Glatiramer acetate is indicated in RRMS at a dosage of 20mg sc. every day [83]. It has shown the ability to reduce relapse rate in RRMS by 29-30% in a randomized phase III trial and to significantly increase the time-span between relapses after a clinically isolated event during a 9-month follow-up [90].

Teriflunomide is a small molecule with immunomodulatory properties that interferes with the proliferation of specific cells by blocking pyrimidine synthesis. Its action on MS stems perhaps from a reduction of circulating T-cells due to this action [91]. It is available at a dosage of 14mg orally, daily. It is indicated in clinically definite RRMS, as it has little impact on clinically isolated syndromes. Similarly, to its first line counterparts, teriflunomide reduces relapse rate by 31-36% in a randomized double-blind phase III trial, and also reduces the rate of disability

progression during a 1-year follow-up. It also drastically reduced CNS lesions when verified by gadolinium-MRI [92].

Dimethyl fumarate is another small molecule similar to teriflunomide with both immunomodulatory and anti-inflammatory actions. Its mechanism of action stems from the activation of transcriptional pathways relating to nuclear factors, such as nrf2. It is available at a dosage of 240mg, that is taken twice per day orally. It holds a similar indication to teriflunomide, being only implicated in RRMS. It showed the reduction of the risk of relapses by 44-53% compared to placebo after 2-year follow-up [93], and by 24% compared to glatiramer acetate, another first-line agent, with similar 2-year follow-up. It also reduces disability progression and greatly decreases lesions verified by gadolinium-MRI [94].

These first line DMTs should be the primary source of treatment in RRMS, SPMS and PRMS as they have shown adequate effectiveness and the ability to retard disease progression and to drastically improve the quality of life of patients. Therapy regimens can be changed between first line DMTs when tolerability issues arise, or the presence of neutralizing antibodies when in therapy with interferon. However, if these DMT do not control the rate of relapse in MS, or RRMS appear to continue progression towards SPMS a switch to a second-line DMT should be considered [83]. Approved second line agents are fingolimod, cladribine, mitoxantrone and the monoclonal antibodies natalizumab, alemtuzumab and daclizumab [94].

Fingolimod was a previously considered first-line treatment option for RRMS. It is however considered a first-line option in the United States [83]. It actively antagonizes the sphingosine-1-phosphate receptors (S1P1, S1P3, S1P4, S1P5) of T-cells promoting a reduced accumulation in the CNS, resulting in an overall improvement in the inflammatory state of the CNS. It should be noted however, that in order for fingolimod to achieve its biological function it requires phosphorylation by sphingosine kinases [95]. It is available at a dosage of 0.5mg once per day, orally. It showed a 48-55% decrease in risk of relapses with a reduction in disability progression in about 20-30% of patients during a 2-year follow-up of the patients [96]. This particular drug has an important feature relating to the presence of varicella zoster, which can result in a fatal activation of the virus. It is therefore recommended to actively look for the infection and for patients undergoing therapy with fingolimod to be vaccinated against this virus [94].

Cladribine, a drug commonly used for the treatment of hairy-cell leukemia is a recently approved treatment option for both RRMS and SPMS that did not respond to first-line options [97] It exerts its action by in vivo conversion to a compound structurally similar to adenosine deaminase, which is a compound that has been found to cause lymphopenia (mostly T- and B-cells, little impact on NK cells) [98], Cladribine is available as 10mg, take once or twice orally for 4-5 days during two weeks in a year with the intention of achieving a cumulative dose of 3.5mg/kg body weight over 2 years. It was initially enrolled in phase I/II studies for PPMS, but ultimately achieved better results regarding RRMS. It reduced relapse rates both during and after treatment discontinuation. Phase III trials showed lower rates of relapse compared to interferon (78-80% vs 60%) as well as lower risk of disability progression. It also greatly reduced the loss of brain volume in MS patients and contributed to the decrease in gadolinium-MRI lesions during a 2-year follow-up [99]. Cladribine also showed the potential to delay a first demyelinating event into clinically definite multiple sclerosis in a phase III trial [100].

Mitoxantrone, similarly an antineoplastic drug, is an approved second-line treatment for all forms of MS, except PPMS. It is a drug that targets rapidly developing cells, affecting DNA synthesis through inhibition of the enzyme topoisomerase [101]. It is available at calculated doses per body surface area of 12mg/m2 intravenously (i.v.) and as proven the ability to improve symptoms and disease progression in RRMS and SPMS, showing a decrease in the risk of relapses by 65% and a reduction of disability progression by 66% [102]. It should however be used as a last restore in case of tolerability issues to other drugs or failure to respond to therapy, due to its toxicological features [94].

Natalizumab is a monoclonal antibody that aims to control the inflammatory process not by acting directly on the function that T-cells hold, but by preventing their ability to reach the CNS. It binds an adhesive molecule named VLA4, by its α4-integrin subunit of α4β1- and α4β7-integrins on the surface of the cell, preventing its association with the target receptor at the BBB, effectively impeding diapedesis across the BBB [103]. It is available at a dosage of 300mg i.v. every four weeks. It has shown a reduction of relapse rate by 68% during a 2-year follow-up, whilst also reducing the risk for a relapse by approximately 59%, with an added 42% reduction in disease progression. It also promoted a reduction of new lesions detected in gadolinium-MRI by 92% [104]. Similarly, to therapy with interferons, natalizumab may lead to the development of neutralizing antibodies which can greatly hinder therapeutic effectiveness, that should prompt a switch in therapy.

Alemtuzumab is another monoclonal antibody that targets a shared receptor (CD52) expressed by most immune cells, leading to a global decrease in immune cells throughout the CNS [105]. It should be noted that in certain regions of the world alemtuzumab is considered a first-line treatment. It is available at 12mg daily for the first five days of treatment, followed by yearly administrations of 12mg daily for three days. It has shown a decrease in relapse rate by 55% with a reduction in lesions shown in MRI by 62% during a 2-year follow-up [106].

Lastly, daclizumab is similarly a monoclonal antibody that blocks a receptor (CD25) targeted by pro-inflammatory interleukins, namely IL-2 leading to a decrease in the activation of T-cells [107]. It is available at a dosage of 150ms, sc. once per month. Daclizumab, however, has not had quite promising results as a single agent preventing relapse rates, but has shown moderate effectiveness when co-administered with interferon. Its administration should thus be saved for when a lack of response has been demonstrated after three or four different types of treatment.

Siponimod (previously known as BAF312) is a derivative of fingolimod that was very recently approved in 2019 for the main treatment of SPMS, and certain cases of RRMS. It is a pivotal drug as it may establish itself as the landmark treatment in patients with a secondary progressive phenotype of MS, after previous longstanding relapsing-remitting cycle. It differs from fingolimod due to not needing in vivo phosphorylation. It is available at a dosage of 0.25mg and 2mg orally, the 0.25mg being used as a dose escalation regimen through six days to achieve the maintenance dose of 2mg daily. Phase II trials demonstrated a reduction in 90-94% of gadolinium-MRI injuries and a reduction in the relapse rate of patients, however, with no significant impact in disability progress. Phase III trials showed a relapse risk reduction of approximately 21% [108]. Due to its still fairly recent arrival in the market, its correct position in therapy has still not been assessed. It should however be considered when patients transit from RRMS towards an SPMS phenotype, due to the drugs main action towards SPMS.

It should be noted that almost all of these treatment options (both first- and second- line treatments are ineffective in controlling PPMS or halting its rampant progression. Certain drugs like azathioprine or cyclophosphamide can be used to attempt a symptom management approach, but the impact in the disease is little-to-none and the impact in relapse rate is negligible [109]. In fact, only one approved treatment has been demonstrated to show activity and promise against PPMS, and a fairly recent approval, having been granted breakthrough approval by the FDA. Ocrelizumab, an FDA approved drug for PPMS is a monoclonal antibody

that selectively binds CD20, a vastly overexpressed receptor of B-cells and drastically reduces the amount of activated autoimmune B-cells in circulation [110]. Ocrelizumab is administered twice per year at a dosage of 600mg i.v., given as two separate infusions of 300mg split among two weeks. Ocrelizumab showed a reduction in disability progression of about 6% in a 5-month follow-up study, whilst also showing a decrease in the number of lesions observed in MRI compared to placebo [111].

Thus, currently approved treatments are a dime a dozen for MS, amongst approved therapies, there is nothing that directly targets remyelination or is able to stimulate mobilization and differentiation of newer OPCs towards mature oligodendrocytes in lesion sites. It is therefore imperative to look again towards the research booth, and to attempt to tackle MS through other pathways that aim to promote either a cure or a restoration of the myelin state of the CNS.

2.1.2 Novel approaches towards multiple sclerosis

Considering the previously described facts regarding approved therapies in MS, novel approaches towards therapy in MS are not only desired but also vital, as cures still seem a way off, and most approved therapy as the potential of eventually losing effectiveness. Thus, plenty of different approaches are researched and published every year, with promising results and the possibility of clinical translation. New molecules working on new therapeutic targets are currently in pre-clinical development or have made it to clinical trials. Therapies based on the use of autologous or allogenic cells have been presented and shown their potential in animal models, as well as gene editing therapies. Nanotechnology has also made a resounding impact due to the versatility of the delivery systems and the ability to reuse previously discarded treatment options due to their bioavailability.

2.1.2.1 – New active drugs with impact on multiple sclerosis

In MS, the lack of both an effective cure and potential lack of therapeutic effectiveness in some variations of certain drugs, drives forward the necessity to develop new entities that can correct these faults.

Ozanimod (RPC-1063) is a derivative of fingolimod, which shares its immunomodulatory features. It similarly blocks sphingosine-1-phosphate receptors (although only S1P1 and S1P5) but does not require phosphorylation to be active.[92] Ozanimod demonstrated the ability to promote a reduction by 84-89% of gadolinium-MRI lesions compared to a placebo during a 5-month follow-up. 83-89% of patients that were selected for the trial experienced no relapse drug in the study observation period [112]. Safety trials regarding potential cardiovascular toxicity of ozanimod demonstrated that with adequate dosing ozanimod is safe to use [112]. Ozanimod very recently underwent phase III clinical trials, whose results should be published soon, and was submitted to the FDA for the treatment of RRMS by its producer, Celgene [Clinicaltrials.gov identifier NCT02294058].

Ponesimod (ACT-128800) is another derivative of fingolimod with a clinical action similar to ozanimod, that does not require phosphorylation in order to achieve its active form [90]. Ponesimod has demonstrated potential in IIB clinical trials where it showed a reduction in gadolinium-MRI lesions at a 5-month follow-up compared to a placebo [113]. In a phase 3 clinical trial compared to teriflunomide, it demonstrated a higher ability to reduce relapses and brain volume loss, resulting in its recent approval by the FDA in the USA [114].

Bruton tyrosine kinase inhibitors are a novel class of drugs, which inhibit a key tyrosine-kinase from the B cell receptor signaling pathway, preventing their rampant activation [115]. Although initially developed towards lymphomas, they have garnered attention towards therapy for MS, and have indeed completed a phase II clinical trial completed mid 2023 (NCT05119569), progressing towards two identical follow-up phase III studies (NCT04586010 and NCT04586023). It will also be compared to ocrelizumab in another trial (NCT04544449).

Older agents that are approved for other therapies can be discussed, as well as new targets that could make a resounding impact in therapy. Rituximab is an example of a well-established therapeutic agent in certain liquid tumors and rheumatoid arthritis that has been recently tested in its potential as an immunomodulator for MS. It works similar to the approved PPMS medication, ocrelizumab, blocking CD20 in B-cells, differing only in their molecular composition [116]. Rituximab has demonstrated in phase 2 trials the ability to reduce annual relapse rates (20,3% vs 40%) at a 1-year follow-up and a reduction of 93% in gadolinium-MRI lesions. It did however not show any statistical difference regarding the control of disability progression [117]. It is currently undergoing a comparison study vs dimethyl fumarate as a phase III clinical trial,

with hopes of achieving an FDA approval for Rituximab, within 2024 [ClinicalTrials.Gov identifier: NCT02746744]. It can currently, however, be used as an off-label prescription for MS [118].

Regarding novel attempts at remyelination, not many drugs are currently undergoing clinical trials. Bazedoxifene (NCT04002934), an estrogen modulator currently used for cancer therapies and osteoporosis in the USA had been considered as a potential remyelinating agent and has been currently undergoing clinical trials to verify if it is able to promote remyelination in RRMS The study is planned to be completed by 2024 [119].

A combinatory scheme of clemastine and metformin [120] (NCT05131828) is currently under study as well to verify if the synergistic action of both drugs can actually lead to an increase of remyelinated neurons at the CNS. The Phase IIa clinical trial is currently underway, with results expected towards the end of 2023.

2.1.2.2 – Cell-based therapy

Although many new drugs come into the market, they still only accomplish at most a delay of MS progression, without being able to repair the CNS or to promote a refurbish of the CNS microenvironment. Furthermore, adverse effects of the medications are common, and can be quite debilitating. Due to the pathophysiology of MS, the use of cell-based therapy may prove to be an effective pathway at achieving a better control of the disease and its progression or even adequately regulating the dysregulated systems of the body to attempt to restore homeostasis [121].

Cell-based therapies have a number of interesting approaches regarding MS. One of the first approaches applied and the one currently most advanced in clinical trials is the use of an autologous transplant of hematopoietic stem cells after a “reset” of the immune system. The rationale behind this method stems from the possibility of completely resetting the immune system into clearing the auto-reactive immune cells, allowing the proliferation of adequate, competent immune cells [122]. Commonly in MS patients, as stated there is an abundance of unregulated T CD8+ and a decrease of effector action by Treg cells, as well as an abundance of mucosal-associated invariant T cells. These are notoriously pro-inflammatory and are

responsible for the production of multiple cytokines that contribute and exacerbate to MS pathology. Post hematopoietic stem cell transplant however, a shift is observed in T-cell population, towards higher numbers of T CD4+ and normalized T-reg function [123]. However, the risk of the resurgence of an autoreactive state of the immune system, and a re-shift towards pro-inflammatory microenvironment is still possible, even with adequate following and a strict treatment regimen, usually leading to a secondary progressive state [124].

Retrospective studies regarding stem cell transplantation show some promising results regarding MS, although they were previously reserved for progressive cases of MS, rarely to RRMS. Results from these retrospective studies found essentially positive outcomes regarding disease activity and remission-free survival periods at 5-year marks [125, 126]. As a result, the use of this treatment option has reached phase II trials, where most trials reported improvements in the disability score of patients as well as improved imaging outcomes compared to standard DMT regimens [127, 128]. Progress towards phase III trials regarding treatment efficacy is still required, although no regimen has yet to reach it yet. Positive factors that impact response to autologous hematopoietic stem cell transplantation are a non-progressive form of MS (RRMS), younger age and few first-line immunomodulatory therapies [126]. Patients that are expected to undergo this therapy regimen should be monitored closely to avoid MS-related complications during preparation for the treatment.

Other types of stem cells have been studied and are currently in preclinical/clinical development. Neural stem cells (NSCs) are multipotent cells obtainable from the CNS of adults. They primarily differentiate into glial cells or neurons [129]. Primary studies on the model of choice to replicate in MS in laboratory animal, experimental autoimmune encephalomyelitis (EAE) show that a direct intraventricular injection of these cells can ameliorate its clinical signs and reduce CNS inflammation [130]. Also, these cells were able to migrate to lesion locations in the same animal model, and to promote their remyelination [131]. Furthermore, they have been linked with the ability to promote the repair of the MS-leaky BBB, one of the main problems in MS, and a decrease in the expression of adhesion molecules that trigger lymphocyte migration to the CNS at the BBB [132]. Lastly, these cells have also been attributed to the production of proteinases that degrade mediators that contribute to axonal degeneration, preventing neuronal death and axonal recession, promoting axons to grow past the glial scar formed at a lesion site [133]. Currently, NSCs are still at a preclinical stage. However, a phase I open-label clinical trial has been performed and demonstrated that intrathecal administration

is safe and tolerable and can result in improved disability scores in patients with progressive forms of MS [134]. Further research and potentially phase II and III trials could help situate NSCs in MS therapy regimens.

3- Central nervous system barriers to drug delivery

The CNS is the most vital organ system of the human body and is responsible for the control of practical all higher functions. Due to this, there is a necessity by the body itself to shield the CNS from the blood through the presence of highly specialized barriers, that prevent the permeability of most compounds throughout to the CNS.

3.1- The blood-brain barrier

The BBB is a physiological barrier with unique features that promotes the protection of the brain, in order to maintain its homeostasis. It acts as both a physical and chemical barrier, firmly regulating the transport of molecules and cells to the brain, hence providing optimal conditions for neuronal activities. The BBB blocks the passage of harmful agents, such as toxic pathogens and compounds, however, ensuring that essential metabolic molecules, such as nutrients, are not excluded. Thus, the biologic function of the BBB should never be compromised in order to ensure a harmonious communication between the brain and the systemic circulation [135, 136].

The endothelial cells of the BBB are arranged as a relatively thin monolayer (Figure), regulated by astrocytes, pericytes, microglia and the basal membrane, which is a non-cellular component that promotes the fixation of the BBB in place. These cellular and non-cellular components influencing the BBB are known as the neurovascular unit [137].

The endothelial cells of the BBB are transiently regulated by most of the components of the neurovascular unit. However, these cells are constantly being modulated by different signals produced by both astrocytes and pericytes that induce, maintain, up-regulate or down-regulate some features of the BBB and hold plenty of other regulatory functions within the BBB contributing to its function [137, 138]. This led to the proposed theory that strategies targeting

these cells can open the BBB, allowing a more effective delivery of nanoparticles to the CNS. The main adversity to the targeting approach of these cells could possibly be due to the experienced toxicity of a non-selective and possibly widespread opening of the BBB, as will be presented.

The basement membrane, although not considered properly a cellular component of the BBB, allows the anchoring of BBB cells in place (pericytes and perivascular macrophages), contributing to the correct structural organization of the BBB and, subsequently, aid it in its functions [139]. It consists of compounds that promote its structure (i.e., collagen, elastin), proteins (i.e., laminin, fibronectin) and proteoglycans (i.e., perlecan, agrin). Its constitution will also contribute to the trans-endothelial electrical resistance of the BBB, it will affect pericyte migration and differentiation [140] and furthermore it can affect the BBB permeability, as a lack of certain components such as laminin can be directly associated with lower astrocyte end-feet association with endothelial cells, a lower number of pericytes and an overall decrease of certain tight junction proteins [140]. Lastly, some components of the basement membrane can also regulate certain processes and signals between astrocytes/pericytes and the endothelial cells of the BBB [141]. Nonetheless, the role of laminar blood flow through the BBB, which generates a certain force at the apical surface of the endothelium, denominated shear stress, has shown to have a role in both BBB-like phenotype induction and in the maintenance of a healthy and correctly functioning BBB. A study by Cucullo et al., demonstrated that an exposure to blood flow promoted increases in RNA levels for genes encoding for tight junctional proteins and cell-adhesion proteins, compared to a similar model that wasn't exposed to blood flow and failed to develop barrier-like properties. Not only this, but the same study also remarked that shear stress promoted the increase of the expression of genes encoding several efflux pumps. Furthermore, it was also presented that exposure to shear stress also positively modulated the expression of ionic transporters (Ca2+ and K+) and solute transporters (such as the various glucose transporters). Thus, this breakthrough study highlighted that importance of shear stress, showing that it positively affects various features for which the BBB is known, and necessarily has to execute with perfection [142].

Astrocytes are represented at the BBB by their end-feet and cover almost up to 90% of the BBB endothelium. Astrocytes are one of the main types of glial cells, making up the overwhelming majority of the cells that exist in the brain [143]. Astrocytes were seen as supportive glial cells at the BBB, but recent research has shown that their role is undeniably

vital to a correct function of the neurovascular unit [137]. These cells are the main inductors of the special phenotype endothelial cells of the BBB acquire and envelop almost the totality of the BBB endothelium through their end-portions known as their end-feet [144]. Thus, astrocytes act as the main regulator of the barrier functions of the BBB, mostly through their secretion of soluble factors, due to the lack of direct physical contact with endothelial cells. However, astrocytes by themselves do not induct all these features in endothelial cells during its development, as an early-BBB seems to appear before astrocytes are fully differentiated in some species [145]. Subsequently, their development and differentiation is triggered by the endothelial cells of the BBB [146].

Astrocytes are key regulators of the brain availability of certain nutrients [147], controlling the expression of the receptors and transporters that recognize these nutrients [148]. Moreover, they are able to tune the permeability of the BBB through tight junctions (TJs) and efflux pumps modulation [149, 150].

Without directly considering endothelial cells, but no-less important, astrocytes are also responsible for the development of neurons, controlling neurotransmitter levels and managing immune reactions within the CNS [139]. Taking this in account, their correct interaction with the endothelial cells is unequivocally essential for the global function of the neurovascular unit and the integrity of the BBB [137].

In vivo studies comparing endothelial cells cultured with astrocytes and without astrocytes showed a direct decrease in the permeability of these cells, highlighting that astrocytes have the ability to tighten the tight junctions between endothelial cells and to reduce the area of gap junctions [150, 151]. Additionally, astrocytes also have the ability to up-regulate several transporters expressed by brain endothelial cells, where some of which hold a key role in delivery pathways across the BBB, such as the GLUT-1 expression and glutathione transporters [152]. Similarly, the expression of receptors thoroughly used in receptor-mediated transcytosis, such as LDLR and TfR is also tightly regulated by astrocytes [152]. On the other hand, astrocytes are also able to induce the expression of P-glycoprotein multidrug resistance proteins expressed at the BBB [153]. Considering the use of receptors or transporters is currently the main and most efficient pathway for drug delivery systems across the BBB [154, 155].

Some chemical segregations mediated by astrocytes, such as glutamate, aspartate, nitric oxide and serotonin can cause an opening of the tight junctions between the endothelial cells [137, 152, 156]. However, some chemical or enzymatic signals from astrocytes can greatly diminish the BBB permeability, such as the production of angiotensin-converting enzyme-1, which ultimately allows angiotensin-II binding to AT1 receptors on endothelial cell allowing for the stabilization of these cells, or angiopoietin-1 that increases tight-junction effectiveness through an up-regulation of junctional proteins [149].

Not only is the interaction with endothelial cells vital, but astrocytes have also shown the ability to regulate the differentiation of the conformation of pericytes, preventing them from altering their natural configuration to a configuration that promotes BBB opening and subsequently leakage [157].

Finally, astrocytes are also responsible for the production and the correct association of basement membrane compounds, such as laminin, N-cadherin, neural cell adhesion molecule and fibronectin or some catabolic proteins such as metalloproteinases. This is a function that astrocytes share with pericytes, although pericytes seem to have a higher importance in this matter [158-160]

Pericytes are located on the smaller caliber blood vessels, in close association to endothelial cells throughout the whole body, but with a particularly high expression on the CNS. Due to this, their expression across the BBB has been subject to a lot of research as result of their role regulating the maintenance and permeability of the BBB, and the stability of the blood vessels at the BBB. Their presence is not required for the endothelial cells to develop BBB-specific features, but their absence will result in an abnormally leaky BBB, unable to filter all compounds as it normally does and ultimately lead to CNS disorders that can impair a normal life [161]. Pericytes can also limit BBB transport through their ability to phagocytose certain compounds after the compounds have successfully crossed the endothelial barrier, ultimately preventing these compounds from disseminating through the CNS [162].

Unlike astrocytes, there is not a consensus on the surface of the BBB that pericytes are in contact with and subsequently cover, with reports varying from 22% to 99% [163]. As astrocytes, pericytes play a role in the regulation of the tight junctions between endothelial cells of the BBB, because of their close association with them [164]. When co-cultured with astrocytes and endothelial cells, in a triple culture exam, pericytes offer a substantive increase

in the trans-endothelial electrical resistance of the endothelial cells, further suggesting that a three-way interaction between astrocytes and pericytes is vital for an adequate BBB function [165]. Pericytes can also inhibit both immune cells from crossing the BBB and certain molecules that increase vascular permeability, which is a negative point for the delivery of nanoparticles concealed within macrophages or monocytes [166].

Furthermore, pericytes can express efflux pumps like P-glycoprotein and multidrug resistance proteins, acting themselves as key team-players to remove foreign compounds from the BBB area, assisting endothelial cells and astrocytes [158]. Also, a study by Armulik et al., showed that lack of pericytes increases BBB permeability, due to higher transcytosis, which suggests that pericytes have a role in the low transcytosis activity that is observed in the brain-endothelial cells [167].

Lastly, pericytes have also been reported to synthesize components present in the basement membrane such as collagen, fibronectin, glycosaminoglycans, elastin and laminin. Pericytes also have the ability to directly stimulate the endothelial cells of the BBB to produce these components themselves, further showing that these cells hold a direct role on the regulation of the neurovascular unit (Figure 1.3) [168].

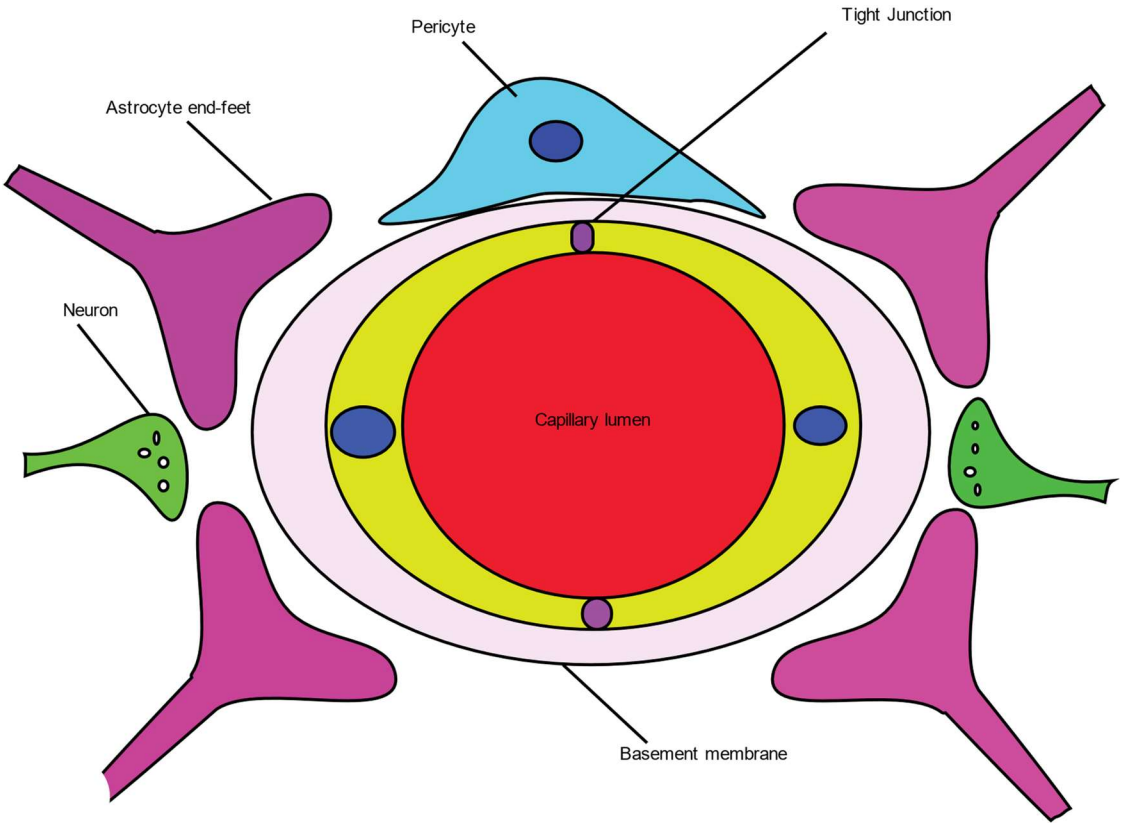

Figure 1.3. The constitution of the BBB and the neurovascular unit and their overall architecture and interactions with the brain capillaries that will be responsible for the blood flow around the CNS. Reproduced, with permission from [169]

Regarding the BBB, its highly-specialized endothelial cells are characterized by highly-limiting TJs and adeherens junctions (AJs), little to none rate of pinocytic internalization and expression of efflux pumps [170, 171]. TJs are a cellular domain mostly made up of integral membrane proteins, such as occludins, claudins and junctional adhesion molecules. These proteins promote a close interaction with other proteins responsible for cytoplasmatic scaffolding, such as actin or zonula occludens (ZO-1, ZO-2, ZO-3) that aim to prevent paracellular leakage of compounds in between the endothelial cells [172]. These TJs provide the main difference between endothelial cells of the BBB and other tissues, as they allow the transendothelial electrical resistance at the BBB to range between 1500-2000 Ω.cm2, as opposed to values of 3-33 Ω.cm2 reported in other endothelia [173]. The AJs are mainly structural, thus promoting

tissue structural support and cellular adhesion. AJs have been described as central determinants in promoting the development and formation of the TJs [174].

The main metabolic defenses of BBB endothelial cells are attributed to the efflux pumps, such as P-glycoprotein, multidrug-resistance proteins, and other ATP-binding cassettes, with a specific location in the luminal membrane. Other defense mechanisms include the considerable expression of cytochromes P450 (CYP450) that rapidly metabolize the molecules that may unduly infiltrate the BBB [175, 176]. Although their expression is lower compared to the liver, CYP450 and other proteases still hold a key role regarding the metabolization of compounds at both the BBB and the brain parenchyma [177]. Astrocytes and pericytes have shown expression of these metabolic defenses, especially efflux pumps, in order to ensure the elimination of undesired compounds.

Considering all the previously mentioned features it is of no wonder that the BBB is extremely selective on the therapeutic compounds that can adequately cross its structure, and it is clear that the BBB only allows the translocation of specific compounds, through specific pathways (Figure 1.4). These include 1) direct paracellular pathway, in which the compound directly transverses the TJs (only applicable to polar solutes); 2) diffusion transcellular pathway, in which the compound directly crosses the cell (only applicable to small lipophilic and gaseous molecules); 3) adsorptive transcytosis, when a compound is internalized through a direct electrostatic interaction with the endothelial cell surface, triggering a endocytosis process; and 4) carrier-mediated transcytosis, in which a receptor interacts with a specific ligand, triggering internalization and transport towards the abluminal side of endothelial cells [178, 179]. Due to this, it is clear that conventional delivery routes are not the most adequate methodology to exploit considering brain drug delivery, giving room to alternative drug delivery options.

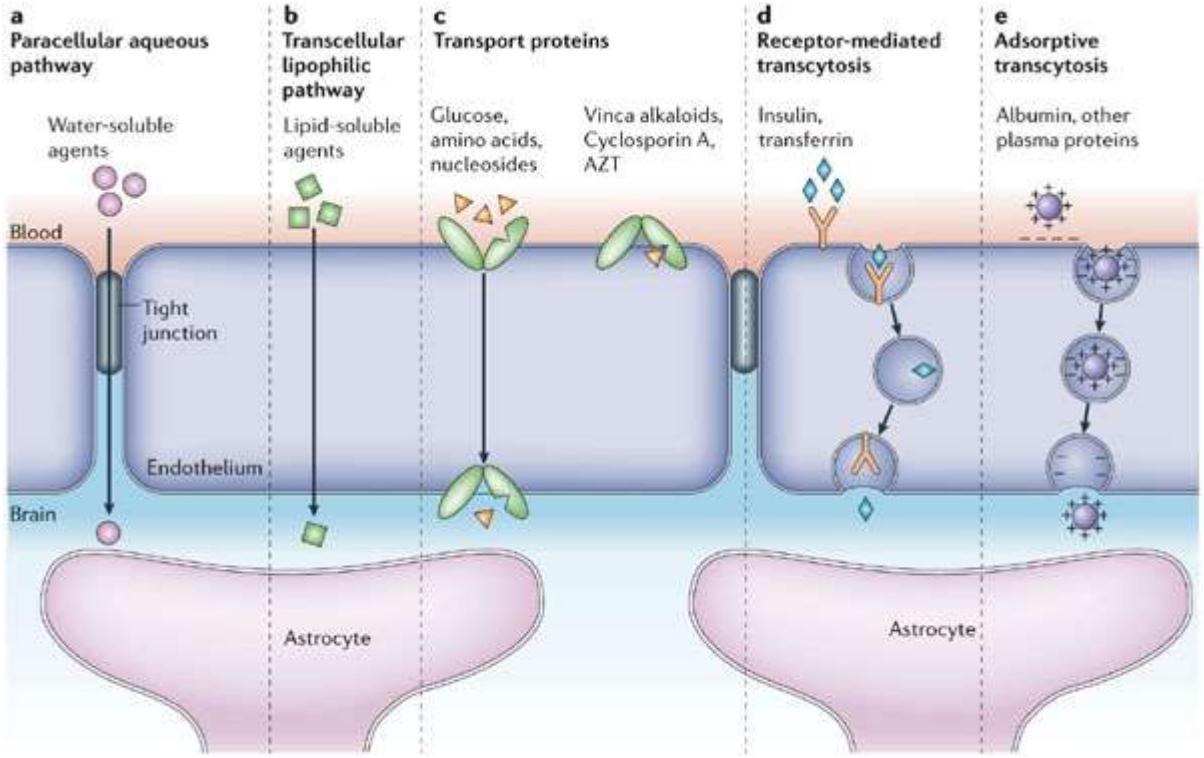

Figure 1.4. illustrating the main transport pathways that occur at the BBB. Pathway a. is seldom exploited as it requires the transient disruption of the tight junctions previously to delivery, a relatively dangerous procedure with regards to the integrity of the BBB. Pathway b. represents the pathway most free drugs use to permeate the BBB. Pathways c., d. and e. are the ones exploited to deliver therapeutics across the BBB, after respective modifications, adding a specific peptide or antibody to adequately target the transporter or receptor previously decided upon. Some of the main receptors and transporters used are also expressed in the figure. Reproduced with permission from Nature Publishing Group© (2005).

3.2- The blood-cerebrospinal fluid barrier

After crossing the BBB, the drugs face a second barrier, the blood-cerebrospinal fluid barrier (B-CSF). Unlike its other barrier counterpart, the B-CSF is relatively permeable to most drugs.

After crossing to the cerebrospinal fluid (CSF), the drugs will cross into the brain parenchyma, through diffusion. However, the diffusion process seems to be very slow, usually resulting in much higher concentrations of free drug in the CSF than in the brain parenchyma. Therefore, due to the accumulation at the CSF, toxicity can occur [180]. Thus here, nanoparticles can perhaps hold another advantage, due to the ability to hold within numerous molecules. Also, the CSF can promote drug movement to the brain parenchyma via another pathway, through Virchow-Robin (perivascular) spaces. However, as this pathway has low rates of fluid flow, it is usually not considered important for drug transport to the brain parenchyma [180, 181]. Nanoparticles can also enter the brain parenchyma through the CSF, but preferably exploit the paravascular pial-glial basement membranes pathway [182].

3.3- Routes of administration and their interaction with the BBB

Considering all the limitations presented by the BBB when developing novel therapies to the CNS, systemic drug delivery seems to require very precise characteristics in order to be effective. Due to this, many likely candidates are not fit to undergo the pharmacokinetic processes from regular systemic administration [183]. There is therefore a need to come up with alternatives to try to bypass the BBB to see if these limitations can be overcome.

Convection enhanced delivery through intrathecal administration has been studied as an approach to directly administer larger molecules directly towards the brain parenchyma, most particularly in tumors. It requires a highly invasive procedure, since the catheter needs to be placed in direct contact with the brain region through which drug delivery would occur [184]. Due to this, this approach is seldom used, as the benefits of the procedure need to outweigh the risks associated with doing the surgery and ensuring correct drug delivery.

However, there is another route that is able to effectively bypass the BBB and promote, in theory, better drug accumulation within the CNS, which is through the intranasal route. The intranasal route offers a non-invasive alternative to bypass the BBB, it also offers potentially rapid systemic effects while reducing any debilitating side effects most medicines might have. Recently, more and more studies have been performed to highlight the potential of the intranasal route to deliver therapeutics for the CNS [185] and there has been an approval [186]

of an antibody (Foralumab) through the intranasal route for the treatment of Alzheimer's disease.

The intranasal route (Figure 1.5) is generally considered to have four potential routes in which drug absorption occurs, but it is usually limited to the location on which the drug deposits itself within the cavity [187]. The olfactory route through the trigeminal nerve, due to the presence of the axons of the trigeminal nerve at the nasal epithelium, then carrying the drug in itself through retrograde transport across the length of the neuron, allowing it to reach the brain parenchyma [188]. Another pathway is the respiratory pathway, wherein the drug can diffuse through the cellular layer of the supporting cells at the nasal epithelium, effectively reaching the systemic circulation, however the drugs would then be faced with the BBB again. The last one would be the nasopharynx-associated lymphoid tissue (NALT), wherein the drug is carried to the lymphatic or systemic circulation, and then reaching the brain parenchyma through this pathway [187].

The limitations of intranasal drug delivery stem from the high mucus turnover that occurs at the nasal cavity, which promptly "washes away" most drug residue that is still within the cavity and the presence of high metabolic enzymes that can deactivate any unwanted compounds [189].

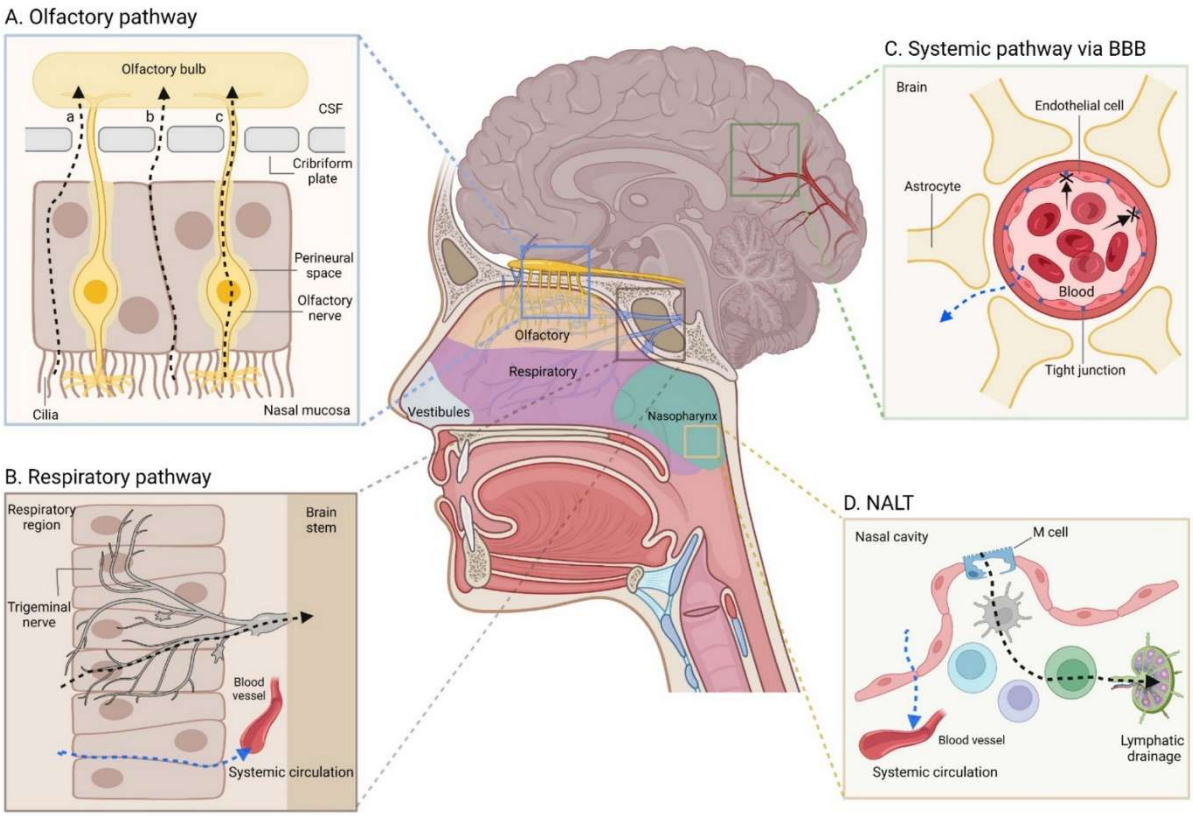

Figure 1.5 – Drug delivery routes through the intranasal pathway in order to reach the CNS. The black dotted arrows in A, B and D indicate the routes that drugs follow when administered, while the blue dotted lines indicate drugs reach the systemic circulation through this route. Reproduced with permission from [187].

3.4- When the bodyguards fail to the disorders: The emergence of a vulnerable brain

The main aim of CNS drug delivery is to promote the delivery of active ingredients towards the brain parenchyma when the brain is afflicted by a specific disorder. However, most published research studies regarding BBB penetration are performed through in vitro models, such as single culture, co-culture, triple co-culture, or dynamic models [190], in supposedly normal BBB conditions. The problem that arises from this approach is that in a plethora of CNS disorders homeostasis is disrupted and further challenges towards drug delivery arise, specific to each condition [191].

Age related neurodegeneration such as Alzheimer's, Parkinson's or Lewy body dementia are all characterized by accumulation of faulty proteins (β-amyloid and α-synuclein, respectively). Through this accumulation, an inflammatory response occurs in the CNS, also affecting the BBB. Downregulation of receptors, such as low-density lipoprotein receptors or the P-gp, which have a role promoting the efflux of the faulty proteins that build-up is an important alteration [192]. Similarly, an increase of IgG can be found near Aβ plaques in the CNS. This could perhaps be explained by the decreased function of the IgG efflux receptor, the neonatal receptor [193]. Furthermore, a decrease in the expression of key TJs proteins is also described, leading to leaky TJs [194]. Perhaps due to the persistent microglia and glial activation (dynamic neurovascular unit members) the harmonious association of the barrier effectively ceases and leads the endothelial cells to fend for themselves, which in turn results in an abnormal, permeable barrier to unwanted compounds. Many cytokines produced by activated microglia can also signal peripheral immune cells to flock to the zone, and increase the expression of integrin, a key protein for the translocation process of immune cells across the BBB [195].

Regarding demyelinating pathologies, such as MS or amyotrophic lateral sclerosis, the global inflammatory state from demyelinating pathologies also leaves its mark on barrier properties. In these pathologies, the main idea stems that the disruption occurring at the BBB is transient, with zones of recurrence depending on the sites of lesions. Due to the constant migration of immune cells through the BBB in demyelinating disorders, which in their own requires interactions with specific adhesion proteins and downstream signal cascade activation [196], promotes destabilization of the internal actin skeleton of the endothelial cells, leading to conformation changes. This in turn affects the association with pericytes, astrocytes and leads to unregulated TJs protein expression effectively reducing TJ behavior, not only at the BBB but also at the B-CSFB [197]. Furthermore, after reaching the brain parenchyma, unregulated immune cells, mainly the cytotoxic ones, produce reactive oxygen and nitrogen species that in turn significantly damage the membrane and TJs association of the endothelial cells or even directly myelin and neurons [198].

Thus, critically thinking about BBB penetration of active ingredients is not sufficient when considering rational drug design or delivery strategies. The pathology that is meant to be treated should be critically evaluated and the possible status of the BBB should be assessed before any treatment regimen is implemented. For example, targeting either transferrin or glucose transporters as a shuttle could prove highly beneficial regarding the delivery rates for

the treatment of tumors due to the overexpression of these receptors by tumor cells. On the other hand, exploiting low-density lipoprotein receptors in neurodegenerative diseases may not be as effective as targeting other receptors due to its significant downregulation. Catering the drug delivery vehicle to the disease in question is therefore essential to improve CNS therapeutic outcomes. Increasing the concentration of the drug towards the CNS is key. However, it can just simply be increased without exceeding the therapeutic index. In order to circumvent this, one of the most exploited ways as of recently is the use of nanotechnology-based approaches, due to the numerous advantages it can confer to an active ingredient.

4- Nanotechnology to bypass the blood-brain barrier

Nanotechnology-based delivery of therapeutic agents involves the use of nanometric particles (dimensions comprehended between 10-1000nm) for the treatment of a particular disease. They can vary on their physical shape and constitution, ranging from liposomes, dendrimers, polymeric nanoparticles, inorganic nanoparticles. These differing nanosystems have different production methodologies and different excipients that make up their structure. Commonly, they will excel at a specific delivery action as opposed to their counterparts, thus requiring an adequate choice by the researchers towards their target [199]. Regarding MS and CNS pathologies as a whole, nanotechnology excels due to their ability to vastly increase the bioavailability of plenty of drugs, antibodies, or proteins to previously unreachable or poor penetration zones of the body [200, 201]. Overall, nanosystems can promote an increase of the bioavailability of a therapeutic agent of interest, whilst achieving controlled release in the desirable location, thus reducing off-target effects resulting in fewer side effects. Furthermore, most nanosystems can be readily synthesized as biocompatible, biodegradable compounds and can be readily scaled-up for industry production [202]. However, the most sought-after feature and the most important to unlock the CNS is the ability to modify the surface of nanosystems to express a specific compound that can selectively target an overexpressed receptor or transporter of the BBB to efficiently cross the barrier with limited resistance (Figure 1.6) [169]. Not only necessarily the targeting of the BBB, but also targeting a specific cell of the CNS, such as oligodendrocytes, is also viable and promotes a several fold increase on the delivery rates. This key feature gives nanosystems an exceptionally wide array of possibilities

regarding the treatment of MS as it can make certain therapeutic agents with proven effectiveness against MS, but poor bioavailability to the CNS, viable candidates for treatment of MS. Nanosystems can also be employed to target immune cells on the periphery due to their direct targeting ability to attempt to control the immune system.

An interesting approach using nanoparticles targeted MS not by attempting delivery towards the CNS, but by inducing specific T-reg cells to control a rampant immune system in autoimmune disorders. Co-delivery of a T-cell epitope of myelin oligodendrocyte protein and a molecule to activate differentiation pathways in T-reg cells showed the ability to effectively suppress clinical signs of EAE via T-reg differentiation and moderation of the immune system [203]. Delivery of agents that promote remyelination has also been demonstrated, where nanoparticles encapsulating leukemia inhibitory factor that were specifically targeted to oligodendrocyte precursor cells to improve CNS penetration demonstrated a significant increase in both the number of myelinated axons as well as globally thicker, healthier myelinated neurons. The BBB was bypassed by promoting the intranasal delivery of the nanoparticles [204]. Stability and efficiency comparison studies were also run between dimethyl fumarate, a commercially available DMT for MS and its encapsulation within solid lipid nanoparticles, wherein the nanoparticles demonstrated the ability to improve the pharmacokinetics of dimethyl fumarate by several-fold [205]. Liposomes with fingolimod were also developed to improve its bioavailability and pharmacokinetics for the treatment of MS [206] Also, liposomes carrying methylprednisolone surface functionalized with glutathione demonstrated to be able to reduce the inflammatory environment experienced at the CNS with increased penetration values compared to free methylprednisolone [207]. Custom synthesized cerium oxide nanoparticles have also been explored in order to confer a certain degree of neuroprotection in the CNS through their anti-oxidant activity. As most immune cells targeting myelin promote their action via reactive oxygen species, this may prove as an efficient therapeutic option to stall the disease. They demonstrated an improvement in clinical scores in EAE models and to adequately accumulate in lesion-abundant zones [208].

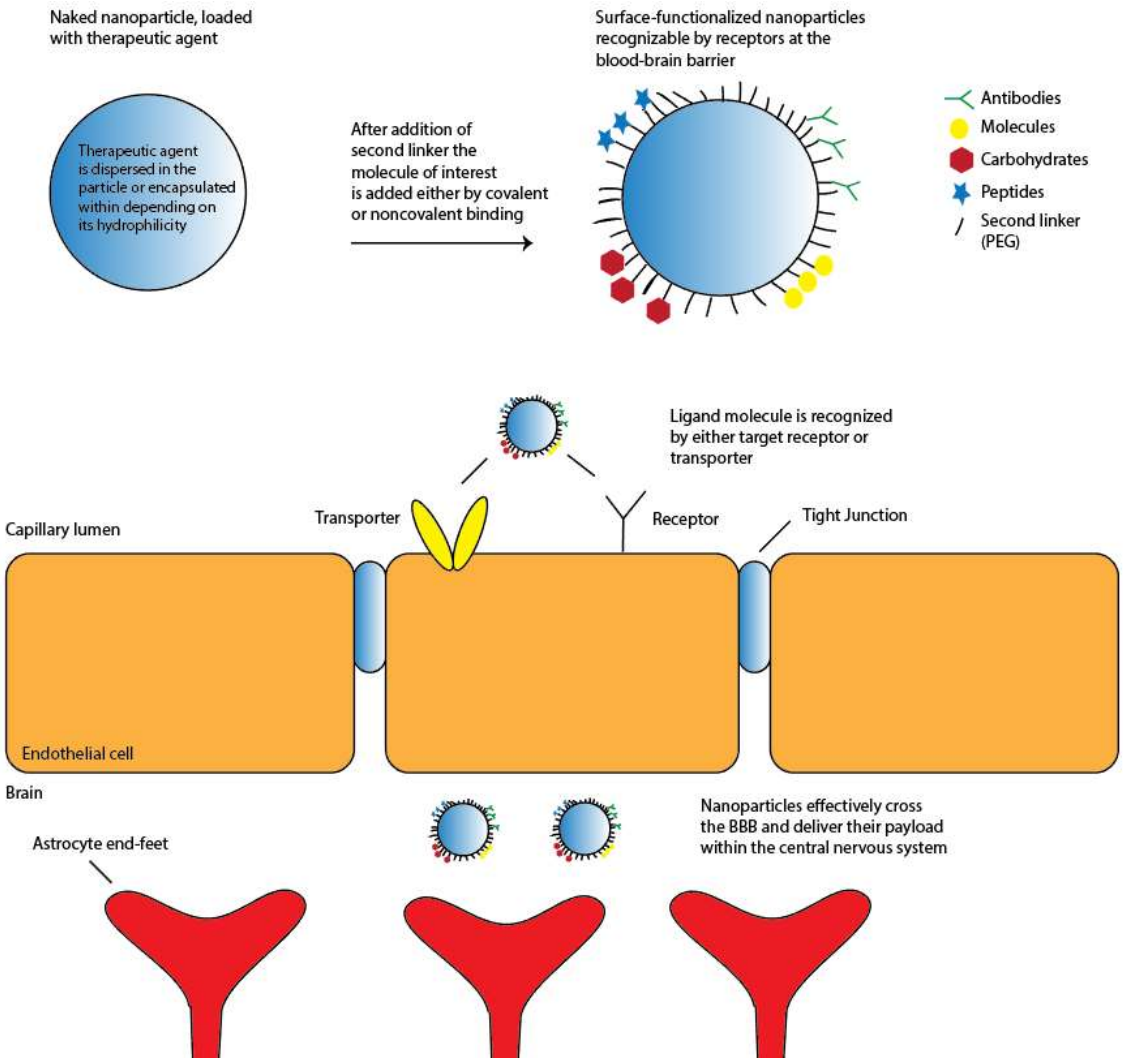

Figure 1.6. Presenting the mechanism beyond the increase bioavailability of nanoparticles through the BBB. Due to the highly-limiting behavior of the barrier, most active molecules are prevented from crossing BBB. However, the expression of receptors and transporters for essential nutrients or compounds to the CNS allow their exploitation by the nanoparticles through surface functionalization. Reproduced with permission from [48].

Not only this, but nanosystems can offer a particular dual action in MS, providing not only a treatment option but also a diagnostic method, in a nanotheranostic system [209, 210].

Also, nanotechnology can eloquently combine with other treatment options presented. Scaffolds, nanogels and hydrogels that act as an extracellular matrix to ensure adequate neuronal function [211], or carbon nanotubes/nanowires that guide axonal regeneration and

promote neuronal regeneration, can be used to restore the CNS to fully functioning conditions. Nanofibers made of biocompatible and biodegradable materials with nerve growth factor also impregnated were exploited in in vivo models. The results demonstrated not only a controlled release of the nerve growth factor, but also that the nerve tube grafted promoted axonal regeneration of the nerves on the zone in which it was grafted. The surrounding area also experienced competent and adequate remyelination. It also did not demonstrate statistically significant differences to the current approved therapy of nerve autografts [212]. Carbon nanotubes have also shown the ability to promote the differentiation of neural progenitor cells into mature cells in in vitro models, whilst also demonstrating neuronal elongation. This synergistic effect can help promote the restoration of a healthy CNS [213]. Also, polymeric artificial cells can be made where a polymeric nanostructure adequately encapsulates a specific bioengineered cell that produces a lacking compound can be implanted in lesion areas of MS resulting in a sustained, rejection-free production of the compound by the safely encapsulated within the biocompatible nanocapsule cell. This method has been exploited in in vivo models of MS [214, 215]. Similarly, the delivery or nucleic acids for gene therapy, such as siRNA [216], can be performed using nanosystems targeted towards the BBB in MS. Using quantum-dots carrying siRNA to silence matrix metalloproteinase-9 (MMP), was an approach exploited having other diseases in mind, but can be adequately implanted in MS. The rationale behind the delivery of siRNA to silence MMP-9 is to target the impending disruption experienced of the blood-brain barrier during the pathogenesis of MS. Since MMP are responsible for promoting a degradation of the extracellular matrix, responsible for holding the BBB firmly anchored in place and a correct association of its cellular constituents, silencing it through gene-therapy allows a study BBB that can be less permeable to foreign pathogens. Quantum-dots promoted several-fold increase of the intracellular penetration of siRNA, whilst the siRNA showed a silencing efficiency of approximately 80% [217]. In a similar approach, lipid and polymeric nanoparticles, surface-functionalized with a transferrin receptor targeting peptide were developed with siRNA aimed to silence P-glycoprotein. Through this suppression, the BBB loses some of its innate resistance towards the delivery of therapeutics, allowing an increase of the permeation of treatment options through the CNS, which could perhaps result in more positive therapeutic outcomes, or even the exploitation of newer therapeutic agents in MS.

Thus, nanotechnology proves it can be a game changer regarding therapeutics of not only MS but also CNS. Their ability to adequately improve the therapeutic potential of a plethora of treatment options whilst maintaining adequate pharmacokinetic and toxicokinetic behavior makes them highly efficient treatment options. The potential to scale-up the manufacturing process is also a tempting feature for novel treatment options. However, despite nanotechnology demonstrating potential, and being lauded as ground-breaking innovation regarding MS, it has yet to live up to its full potential. Design of a proper nanotherapeutic system requires a perfectly harmonious balance between the choice of therapeutic agent, targeting entity and excipients, as well as critical understanding of how it can interact with biological systems. Despite their common safety and the biocompatibility of their excipients, understanding potential immunotoxicity, genotoxicity or cytotoxicity of nanomaterials in an environment as extremely regulated as the brain parenchyma and the CNS as a whole is a challenging and key task to ensure safe translation of nanoparticles to clinical practice. Furthermore, promising advances in the area of nanotechnology related to MS are also held back by the lack of resources by most research centers to develop interdisciplinary work to fully harness nanomedicines potential.

Due to MS multifactorial presentation and the heavy involvement of MS, cell-based therapies and newer biological agents which accomplish immunosuppressive actions in the periphery (ultimately regulating CNS immune system microenvironment) show higher chances of an FDA approval. However, with the ongoing development of the world of nanomedicine it is expected that nanomedicines can fully access their power and tip the scales in their favor regarding the production of capable, MS personalized treatments.

4.1 – Lipid nanocapsules

Regardless of all the efforts dedicated to increase drug accumulation in the CNS, CNS drug delivery still faces many challenges regarding the pharmacokinetics of new therapeutics that must be addressed to achieve satisfactory brain delivery. Here, lipid nanocapsules (LNC) are a type of nanosystem that can be achieved through a simple phase-inversion methodology, without the use of toxic organic compounds that require removal postproduction (such as chloroform or dichloromethane) [218]. LNC are also ideal candidates for highly lipophilic drugs,

since the LNC will allow the drug to be dispersed in external aqueous phases such as water or 0.9% NaCl.

In brief, the method (Figure 1.7) to produce the LNC uses a lipophilic product, mixed with two amphiphilic surfactants in the presence of salt water (Figure 1.7). LNC can be characterized by a lipoprotein-like structure wherein an internal liquid oily phase is stabilized externally by a relatively rigid PEG-based shell. Different excipients can be used to serve as each of the individual constituents of the LNC, where examples of the oily core could be medium chain triglycerides (Labrafac® WL 1349 or Labrafac® CC), ethyl oleate, Labrafil® M1944CS, amongst many others. Normally the amphiphilic surfactant is a PEG-based product, most commonly being a mixture of free PEG and PEG 660 (Solutol® HS-15) while the lipidic surfactant is usually a derivative of lecithin at different hydrogenation levels (Lipoid® S75-3, Phospholipon® 80H, Phospholipon® 90H). With these three-core products present, LNC have been developed with a plethora of different excipients, which should be chosen based upon the affinity that the drug to encapsulate may have towards the oily phase and the stability the surfactant shell will give to the LNC. The shell will also endow the LNC with immune scaping properties, due to the presence of PEG, and grant it resistance to enzymatic degradation.

In order to produce LNC, a three-way balance between the amount of water, oily phase and amphiphilic surfactant must be respected, or the compounds may not assemble into an LNC structure, but rather a micelle or not even associate at all [219]. Thus, typically by keeping one of the amphiphilic surfactants' concentration constant (typically the non-PEG surfactant) and modifying the concentration of the other one, LNC can be obtained by following a specifically designed tertiary diagram that combines the concentrations of the oily phase, the aqueous phase, and the amphiphilic surfactant. A study by Heurtault et al. focused on verifying the best rates to achieve this fine balance between these excipients, as well as a series of investigations regarding a multitude of different proportions and how these behaved when designing LNC [220]. In brief, the concentrations of the oily phase should be between 10-25%, the surfactant should be between 10-50% and the aqueous phase should be between 50-90%, all in w/w, of the initial mixture. These ingredients can be modified within these ranges to tune the properties of the LNC [220]

After the mixture of all the ingredients, a series of heating-cooling cycles are performed, in order to raise the temperature of the mixture above the phase-inversion zone. The phase-inversion

zone can be identified by the detection of an abrupt change in the conductimetry of the mixture. This notorious shift in conductimetry denotes that the mixture has shifted from an oil-in-water emulsion to a water-in-oil emulsion. Normally, there are also organoleptic changes that can be observed at these temperatures. After three or more cycles, at a temperature of 1-3ºC from the beginning of the phase-inversion zone, a sudden quench with cold water (close to 0ºC) is performed, wherein the LNC is assembled in the mixture. Normally, the drug is dispersed previously in the lipid phase, or added before the cold-water quench. It results in a lipid core surrounded by an external shell of surfactants, wherein the lipophilic or amphiphilic drug is safely encapsulated within [218]. This method allows the production of lipidic nanomedicines with stable properties, with optimal size for CNS delivery and a high encapsulation efficiency. Typically, the hydrophobic or amphiphilic drug can be previously dissolved in the oily phase of the mixture and subjected to the heating cycles in order to guarantee its association within the lipid structure after the dilution step.

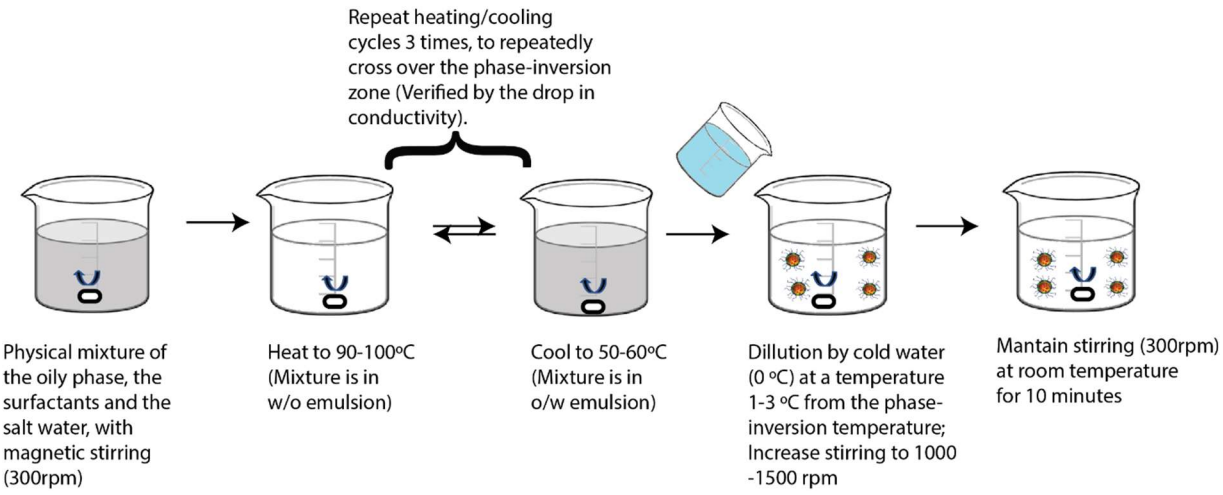

Figure 1.7. Synthesis process of lipid nanocapsules through phase-inversion. Through this process, the LNC are generated using only generally-regarded-as-safe (GRAS) excipients and without the use of organic solvents. Reproduced with permission from [221].

However, regarding the thermolabile nature of some drugs, to ensure their encapsulation with minimal drug degradation due to high temperatures, the drug can be added as a solution a few degrees over the phase-inversion zone, right before the dilution step in the final heating cycle [222]. This is a promising strategy to promote encapsulation of difficult to handle therapeutic

agents, namely proteins or antibodies, that tend to denature when subjected to high temperatures. Inversely, if encapsulation rates are not satisfactory with this approach, by increasing the concentration of sodium chloride on the mixture the phase-inversion zone will be reached at considerably lower temperatures [219]. Regarding the potential scale-up process of LNC, it should be noted that scaling-up a nanosystem can always be very challenging and effectively impairs the development of most clinical candidates. The main limitation is always the production step. Regarding the LNC, one of the challenges going from a pilot-based scale to a large industrial scale is maintaining sufficient energy in the stirring processes that effectively mimic what occurs in pilot-scale formulations. Herein, the study performed by Thomas et al. demonstrates effectively how ibuprofen loaded-LNC behaved when subjected to a scale-up process. The typical long-term stability of the LNC was present in the scaled-up LNC and the physio-chemical properties were comparable to pilot-scale formulations [223].

LNC are a versatile platform when it comes to drug delivery. Due to their tunable properties and readily scaled-up manufacturing process, their use regarding novel therapeutic options should be heavily considered when designing nanosystems. The LNC bears a resemblance to lipoproteins. LNC have some advantages to other lipid-based delivery systems, such as liposomes, due to the lack of toxic solvents used in their manufacture, their vastly improved ability to encapsulated lipophilic drugs and their increased stability in in vivo conditions [219]. Due to the external casing of the LNC being composed of polyethylene glycol (PEG), LNC benefit greatly from prolonged circulation, since they effectively avoid the rapid uptake by the mononuclear phagocyte system, and an ability to transiently suppress P-gp function. Thus, cancers that normally have low therapeutic rates of active ingredients reaching them are prime candidates for LNC-based therapy. As an example of these applications of the LNC, the work carried out by Lamprecht et al. demonstrated where LNC were developed to vehiculate etoposide, the first-line drug treatment for glioblastoma, showed a 5-6-fold increase in concentration in glioblastoma cells compared to the free drug. This is perhaps due to the previously mentioned effect PEG supplies, wherein after the release of the drug payload the PEG shell transiently suppresses P-gp, allowing higher concentrations of intracellular etoposide vs the free drug [224]. To add to this, LNC are also prime candidates to encapsulate other nanosystems and to correct their faults. The work by Resnier et al. showed how LNC encapsulated liposomes that had siRNA dispersed within their membrane [225]. The LNC efficiently altered the properties of liposomes to enhance intracellular delivery in order for siRNA

to develop its action, in in vivo assays. Tsakiris et al. further demonstrated LNC potential, by exploiting the encapsulation of six different active ingredients used in cancer therapy, reporting high encapsulation efficiencies in five of the six proposed active ingredients, and demonstrated their anti-cancer effect in an in vivo colorectal cancer model [226]. Regarding CNS delivery in other pathologies other than cancer, LNC have been developed for neurodegenerative diseases, like Alzheimer's disease. Curcumin was encapsulated within LNC and administered to a mouse model of Alzheimer's disease. The LNC effectively increased the bioavailability of the drug to the CNS and showed neuroprotective properties against the pro-inflammatory environment at the CNS [227]. Lastly, LNC have even been exploited as antidotes for drug overdoses, as their lipophilicity can promote the sequestration of highly lipophilic drugs in vivo, reducing their concentrations to non-toxic levels [228].

Due to the lipidic nature of LNC, they are normally employed to encapsulate lipophilic or amphiphilic drugs. To counteract this, LNC able to encapsulate hydrophilic drugs have been developed. The methodology is based upon the development of an initial w/o emulsion (micelles) encapsulating the drug, where afterwards through the formation of a polymer shell at this interface, the oily phase is then replaced by another aqueous phase, resulting in an aqueous core within the lipid shell [229].

Furthermore, traditional LNC have been demonstrated to be stable at 4ºC for periods of up to 18 months [221, 223, 230], capable of producing a sustained release system, and not rely upon the necessity of freeze-drying in order to achieve high shelf-life. They also maintain their physio-chemical properties, in values comparable to when they were first developed. This here can be essential, as due to their lipid nature and the sublimation properties of the lipids that form the LNC, freeze-drying may prove a challenge. However, aqueous core LNC are not as stable as they tend to experience a burst release of the payload.

The method for the development of LNC can be fine-tuned depending on the use the LNC is intended to have. The review by Huynh et al. presents in detail the impact of LNC modifications on LNC properties [219]. The main findings detailed in this eloquent review are that LNC can be fine-tuned to be produced at nominal sizes, in a highly monodisperse environment by adapting the amount of the oil phase or the amphiphilic surfactant. The concentration of the oily phase primarily impacts the size of the LNC, wherein an increase in the proportion of its oily phase will directly increase their size. The lipophilic surfactant is primarily responsible for

keeping the shell rigid and stable, thus normally being kept at fixed, known to be effective, quantities. Also, the amount of water added in the cooling step can promote a lower polydispersity index (PdI) as it leads to a decrease in the average size of the LNC. The number of heating/cooling cycles performed also tends to improve the PdI of the LNC, but it does not seem to alter the properties much after three cycles [220]. Lastly, the amphiphilic surfactant (typically PEG based) has a major influence on ensuring its stability and ensuring the formulation of LNC structures and no other micellar structures. With regards to the CNS, to achieve optimal delivery rates, nanosystems have to be developed with a particular set of features in mind. One that LNC excel in, is reduced clearance by the mononuclear phagocyte system (MPS). Due to their design, the use of mixtures of free PEG and functionalized PEG, LNC are shielded from the uptake of MPS and have thus increased circulation time, allowing them to interact with the BBB more often, resulting in increased penetration rates. Also, the very same mixture of PEG used in their formation transiently inhibits P-gp activity in cells that actively uptake LNC, promoting an increase of the concentration of the encapsulated drug within them [231]. Naturally, due to the high expression of P-gp at the BBB, the use of these PEG in the development of LNC can significantly increase the accumulation of the LNC in the brain parenchyma. Due to the use of GRAS excipients, LNC are expected to be tolerated by the CNS, demonstrating little-to-none toxic effects [232]. Indeed, in vivo studies corroborate their safety and their suitability for CNS delivery [233].

Due to the structure of the TJs at the BBB, size is key when designing a vehicle for CNS delivery. Nanoparticles that have a smaller size (circa 20-100nm) will more adequately penetrate the limiting TJs of the BBB or achieve paracellular transport [234], but if they are made too small (normally sizes comprehended between 1-20nm)[235], they will be filtered by the kidneys before any adequate accumulation is achieved at the CNS [236]. Also, due to the anionic behavior of the BBB, the presence of a positive charge on the surface of the LNC could improve their penetration, through electrostatic interactions, increasing the cellular uptake of the LNC [237]. However, due to the presence of the PEG facing outwards in the LNC, most LNC systems will have a negative charge. Despite all this, the proportions of each excipient must respect the specific balance in concentration regarding the oily phase, the PEG surfactant and the aqueous phase that was presented in the previous section, by respecting the tertiary diagram proposed by [220], in order to ensure the self-assemble of the components into the LNC structure [218]. Also, the potential to graft on the LNC surface longer chains of Peg (PEG

1500, DSPE-mPEG2000) tends to gift the LNC longer circulation half-life, but can potentially hinder cellular internalization, thus requiring adequate decision-making when considering this approach [238].

Achieving adequate rates towards the BBB is a notoriously complicated challenge that requires a perfect harmony of features on a nanosystem to ensure it can transverse the BBB towards the brain parenchyma. Accumulation rates relying on normal transcytosis and paracellular transport of LNC through the BBB results in very low delivery rates, due to the previously stated features of the BBB. Although in some conditions, LNC can rely upon passive accumulation methods, due to the PEG chains on its surface as stated previously, it will not result in satisfactory delivery rates in most conditions.

Indeed, depending on the mechanism of action of the vesiculated drug within the LNC it is important to carefully plan the best route to explore when delivering drugs towards the CNS. The LNC can follow all standard internalization methods, namely micropinocytosis, clathrin and caveolae-mediated internalization or direct transport through the TJs [202]. Each of these routes has its own drawbacks, and an adequate understanding of the route of the LNC is paramount to understand its biodistribution. Thus, it is imperative that active targeting or novel attempts to circumvent the BBB are exploited. Herein, due to its versatility, LNC can really shine and unleash their full potential as a drug delivery vehicle.

LNC, like most nanosystems, can be functionalized with cell-penetrating peptides that recognize and bind specific receptors and are internalized via receptor-mediated transcytosis [221, 239]. This technique can be performed both by simple adsorption at the surface of the LNC through ionic interactions, or covalently bound by the use of different chemistry reactions. Despite the PEG usually employed to develop LNC not exposing a reactive group to allow for covalent binding of ligands by NHS-EDC chemistry, the surface can be activated with reactive PEG, such as PEG bound with maleimide to allow a receptor-binding ligand to be covalently bound to the LNC surface to target a specific receptor [238]. Additionally, adsorption of certain ligands can be considered to the LNC surface, but due to the weak link it establishes with the LNC, its role in the targeting efficiency and the amount adsorbed at the LNC surface can be questionable [221, 240]. Other approaches to promote active targeting towards the CNS can consist upon the use of magnetized nanoparticles, namely, super paramagnetic iron oxide

nanoparticles (SPIONs) to promote magnetic guidance towards the central nervous system but have not yet been applied to LNC.

Despite the promise these approaches apparently present, there are certain downsides that still difficult CNS delivery. Relying upon the prolonged circulation half-life and transient P-gp suppression by the PEG chains of the LNC is not an optimal way to increase concentration efficiencies, and conjugation efficiencies of ligands with LNC are still challenging procedures. Receptors at the BBB can also saturate quickly, due to internal competition by the endogenous ligand or they can easily be up taken by other cells in different parts of the body that similar expression a high amount of the targeted receptor. Thus, searching for alternative ways to effectively and more efficiently bypass or “beat” the BBB could, and should, be considered in order to attempt to produce clinically acceptable products.

5- Concluding remarks

The CNS is still a hard-to-reach location for novel therapies. The necessity to develop potent drugs that require a perfect partition coefficient in order to cross BBB leads to plenty of breakthrough candidates to be stranded in the bench, effectively hindering therapeutic options. However, through the use of nanotechnology, the necessity for optimal partition coefficient is void, as the nanoparticles can safely deliver the drug encapsulated within them to the CNS, requiring only the tuning of their surface and properties to ensure optimal delivery towards the CNS, without the need of modifying the drug or compromising its therapeutic potency. LNC are a versatile, safe, easy to scale-up platform to consider for CNS delivery. Their safe and solvent free manufacturing process using GRAS excipients makes them a very interesting system for industrial development. LNC emerge as a prime candidate for CNS delivery, primarily due to the ability to fine tune their size and functionalize their surface adequately for CNS delivery, with minimal effort, whilst maintaining high association efficiencies and drug encapsulation rates. The ability to develop these for both hydrophobic and hydrophilic drugs make them a heavyweight candidate to develop formulations towards the CNS. The ability to exploit alternative delivery routes that bypass or temporarily weaken the BBB, with limited effects to the host, have been developed to increase delivery rates. Despite still being certain ways off

from clinical practice due to unspecific BBB opening or the possibility of nefarious effects that are not easy to control, optimization of these practices is still required, and recommended, in order to fully harness the therapeutic arsenal available and tackle CNS ailments properly.

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CHAPTER 2 – OVERVIEW AND AIMS

1.1 – Overview

Amongst acquired demyelinating diseases, the most well-known is multiple sclerosis (MS) affecting approximately 2.9 million people worldwide [7]. MS can arise from a multifactorial pathway, and can express itself in multiple different phenotypes, depending on the progression of the disease [8]. The majority of people will be diagnosed between the ages of 20-50, and will remain with the condition for the rest of their lives, as it currently has no effective treatment option [9]. As it stands, the disease carries a burden of around 85.4 billion regarding both therapeutic options and non-therapeutic limitations from the patients' whose quality of life is affected (data from the USA) [10].

Despite the severe lack of effective therapies for demyelinating diseases, promising molecules/therapeutic approaches are emerging, such as retinoic acid (RA), that have shown to reduce or resolve chronic inflammation and to stimulate remyelination by inducing the differentiation of neural stem cells (NSCs) and oligodendrocyte progenitor cells (OPCs). RA (all-trans or 9-cis-RA) can alter the cellular immune response, suppress the inflammatory response of microglia and act in a pro-regenerative way in the CNS [11]. Studies have demonstrated that RA is able to induce the differentiation of progenitor cells into neuronal cells, suggesting that this molecule is a good candidate for enhancing neurogenesis [12].

The limitation for the treatment of demyelinating diseases is the failure of active molecules to reach therapeutically ideal concentrations in the affected CNS areas. Degradation, hydrophobicity, inability to cross the blood-brain barrier (BBB) and limited uptake by target cell populations are the obvious hurdles[7]. Furthermore, achieving therapeutic concentrations can require administrating higher doses leading to severe off-target effects. Indeed, RA has already been exploited as a therapeutic option in clinical trials and in clinical practice, but its potent *in vitro* effect has not been replicated in humans due to the limitations listed above [13, 14]. Therefore, there is a significant unmet clinical need for the translation of RA towards a delivery system capable of ensuring it reaches therapeutically ideal concentrations at the CNS in order to exert its potent action.

Lipid nanocapsules (LNC) that can reach therapeutically ideal concentrations at the CNS have been developed thoroughly by our research group at UCL [15-17]. LNC are composed of excipients approved by the FDA and the process, based on phase transitions with temperature cycling, are solvent-free, soft-energy and cost-effective to manufacture. The process is simple, easy to scale up, leading to an aqueous suspension of LNC, directly available for therapeutic development. Furthermore, the procedure is adjustable to accommodate to the nature of the drug being encapsulated. To add to this, our group at i3s has demonstrated previously the potential to improve the penetration of nanoparticles through the BBB by the expression of certain peptides and/or antibodies at the surface of nanosystems [18, 19].

To fully elucidate the best possible clinical outcome in the therapeutics, different delivery routes can be tested to verify the potential to accumulate within the CNS. Intranasal administration is a non-invasive route to transport drugs/nanoparticles directly from nose-to-brain avoiding the BBB [20]. LNC have already been successfully used, in pre-clinical models, to deliver GDNF and siRNA via nasal delivery for the treatment of Parkinson's and Alzheimer's diseases [21], respectively.

Therefore, the present work arises from the necessity to further develop novel clinical alternatives for the delivery of compounds capable of not only controlling the uncontrolled inflammatory response at the CNS, but also to restore the myelin of the afflicted areas, contributing towards restoring the balance of an affected brain of an MS patient. We hypothesize that our LNCs are capable of promoting an improvement to the pharmacokinetic parameters of RA and clearly demonstrate an impact both *in vitro* and *in vivo.*

1.2 – Aim and specific objectives

The aim of this project is to develop nanomedicines capable of stabilizing and promoting the repair of CNS myelin under assault by acquired demyelinating diseases. It involves the formulation of LNC capable of encapsulating RA, and co-encapsulating them with SPIONs, while externally functionalizing the shell of the nanocapsules with a TfRp capable of recognizing the TfR at the BBB to promote the accumulation of the LNCs within the brain parenchyma.

The objectives create a unique synergy while being original and oriented towards clinical translation:

1) To develop and characterize relevant physicochemical and technological properties of nanoparticles based on LNCs, co-encapsulating RA with SPIONs while modifying the external shell of the LNCs to express the TfRp in order to yield better rather of brain permeability.

2) To evaluate the safety profile, the specificity and the influence of the LNCs on target cells within the CNS, by demonstrating the efficiency of them in promoting the differentiation of OPCs and resolving the inflammatory activation of resident microglia.

3) To verify the capability of the LNCs to successfully navigate the BBB or the nasal epithelium, resulting in increased rates of accumulation at the brain to ensure better therapeutic outcomes.

4) To achieve a proof-of-concept of the LNCs, demonstrating its safety and CNS accumulation in healthy mice, in order to translate them to a hallmark disease model of EAE, to correlate its *in vitro* properties in an *in vivo* model.

This thesis is organized in 5 chapters. In chapter 1, a comprehensive and exhaustive review of the literature surrounding the theme, carefully explaining the literature behind the theme of the study, and rationalizing the concept of the work, to explain it as an unmet clinical need. In chapter 2, a general framework of the theme is given, as well as the motivations and the aim behind the study. Here, the objectives that we aim to achieve within the thesis are thoroughly specified. Chapter 3 represents the work regarding the development and validation of the physico-chemical properties of the system, with and without surface modifications, as well as demonstrating a clear panel of work on the safety, specificity, and applicability of the LNCs to navigate the BBB and exert its action. In chapter 4 it is described the following steps after the *in vitro* validation, towards obtaining a clear and defined proof-of-concept of the LNCs by comparing two routes of administration in healthy mice, to verify that is lacks any toxicity and has adequate brain accumulation, before demonstrating its impact in a disease hallmark model capable of adequately predicting the impact in MS of the LNCs. To conclude, chapter 5 includes the overall conclusions of the study, and discusses the future outcomes and the contingency measures that can be applied when continuing this work in order to keep perfecting the nanosystem towards clinical approval.

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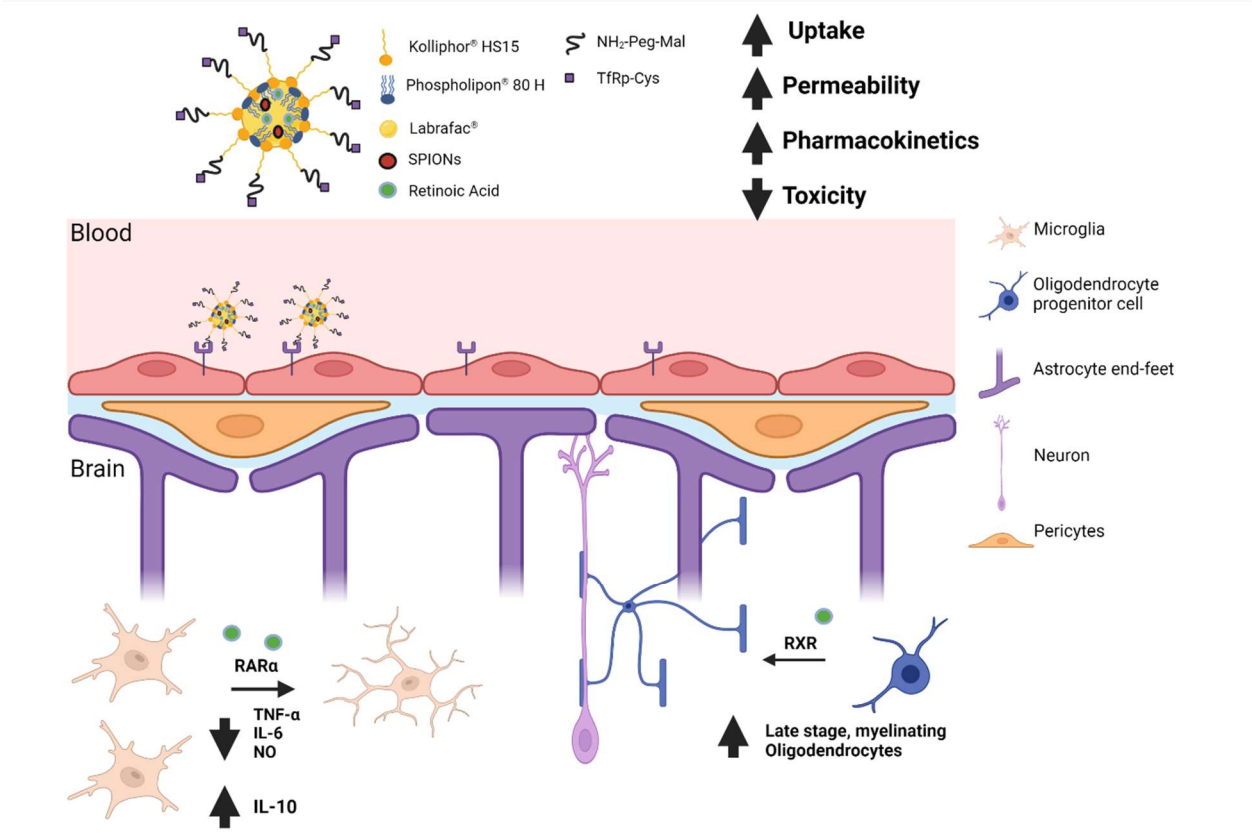
CHAPTER 3 - FUNCTIONALIZED RETINOIC ACID LIPID NANOCAPSULES PROMOTES A TWO-FRONT ATTACK ON INFLAMMATION AND LACK OF REMYELINATION ON NEURODEGENERATIVE DISORDERS



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Functionalized retinoic acid lipid nanocapsules promotes a two-front attack on inflammation and lack of remyelination on neurodegenerative disorders

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Abstract

Demyelinating disorders, with a particular focus on multiple sclerosis (MS), have a multitude of detrimental cognitive and physical effects on the patients. Current treatment options that involve substances promoting remyelination fail in the clinics due to difficulties in reaching the central nervous system (CNS). Here, the dual encapsulation of retinoic acid (RA) into lipid nanocapsules with a nominal size of 70 nm, and a low PdI of 0.1, coupled with super paramagnetic iron oxide nanoparticles (SPIONs) was accomplished, and joined by an external functionalization process with a transferrin-receptor binding peptide. This nanosystem showed

a 3-fold improved internalization by endothelial cells compared to the free drug, showed their ability to interact with oligodendrocyte progenitor cells and microglia, and showed improvements in the permeability through the blood-brain barrier by several fold. The lipid nanocapsules also induced the differentiation of oligodendrocyte progenitor cells into more mature, myelin producing oligodendrocytes, as evaluated by high-throughput image screening, by 3-5-fold. Furthermore, the ability to tame the inflammatory response was verified in lipopolysaccharide-stimulated microglia, suppressing the production of pro-inflammatory cytokines by 50-70%. Overall, the results show that this nanosystem can act in both the inflammatory microenvironment present at the CNS of affected patients, but also stimulate the differentiation of new oligodendrocytes, paving the way for a promising platform in the therapy of MS.

Keywords: Dual Therapy; Nanoparticles; Neurodegeneration; Myelination.

1. Introduction

Demyelinating disorders are characterized by a progressive loss of the insulating layer that enwraps neurons in the central nervous system (CNS), known as myelin, and typically resulting in significantly impaired neuronal communication [1]. These diseases are characterized by a progressive development of lesions with loss of function of critical cellular players in the homeostasis, such as oligodendrocytes, microglia, and astrocytes [2]. Oligodendrocytes in particular are the cells that produce the myelin in the CNS [3]. Oligodendrocyte disfunction or direct myelin destruction are the main issues in demyelinating disorders, such as multiple sclerosis (MS) [4], which typically leads to significant impairments in the quality of life of individuals. MS particularly stems from a multifactorial ethology, wherein the underlying cause is still not fully understood, where a targeted immune reaction occurs against the myelin, leading to the formation of a persistent inflammatory state. Different theories have been put forward, ranging from genetic, environmental, or even infectious due to molecular mimicry. At early stages of the disease, remyelination can occur when oligodendrocyte-progenitor cells (OPCs) are mobilized to a lesion area where focal demyelination is occurring, and undergo differentiation into mature, myelin producing oligodendrocytes [5]. In late chronic states of MS,

oligodendrocyte progenitor cells (OPCs) or oligodendrocytes are not able to restore the destroyed myelin anymore. Additionally, the formation of a glial scar within the lesion sites not only contributes to the failure of the regeneration process but also to further neurodegeneration [6].

Therapeutic approaches currently in clinical practice for MS focus primarily on disease-modifying therapies (e.g., dimethyl fumarate, fingolimod, teriflunomide) that attempt to control the inflammatory environment at the CNS to reduce the rate of demyelination and symptom flare-ups [7][8]. The main limitation behind the lack of therapeutic options that focus on remyelination stems from the poor bioavailability of candidate drugs to the CNS due to the presence of the blood-brain barrier (BBB) [9]. In order to improve therapeutic outcomes in demyelinating disorders, particularly MS, alternative therapies must be considered to fully harness the power of remyelinating agents.

Lipid nanocapsules (LNCs) are a versatile nanosystem that can be rapidly developed by a process devoid of organic solvents that follows a phase-inversion methodology [10]. LNCs are made up of Food and Drug Administration approved excipients for all routes of administration and can promote the encapsulation of a plethora of different therapeutics [11, 12]. The preparation process is simple to scale-up and the LNCs exhibit long-term stability when stored at 4 ºC, highlighting the potential for clinical application of these particles [12]. Due to their lipidic nature, it is also quite simple to encapsulate lipid fluorescent tracers to track the LNC in multiple assays that require particle tracking [13]. Furthermore, the surface of the LNC can be modified to carry targeting agents that promote an increased accumulation of the LNCs in target tissues or cells [14]. With a particular interest in improving the bioavailability to the CNS in the case of MS, targeting the transferrin receptor (TfR) using either antibodies or peptides has been the preferred option, due to the overexpression of the receptor at the BBB [14]. TfR is a promising target since it is exclusively expressed by endothelial cells of the brain capillaries thus preventing significant off-target effects [15]. For instance, a previously reported TfR-targeted peptide (TfRp) (Thr-His-Arg-Pro-Pro-Met-Trp-Ser-Pro-Val-Trp-Pro-Cys) effectively showed the ability to promote polymeric nanoparticles with a significant improvement in permeability through a BBB in vitro model [16, 17] and BBB cell uptake [18].

Retinoic acid, particularly all-trans retinoic acid (RA), is a widely used agent in many differentiation protocols for neural stem cells [19]. Additionally, RA can also promote alterations

in the immune response caused by microglia [20] and act in a pro-regenerative way at the CNS [20]. Studies have already demonstrated that RA can induce the differentiation of neural stem cells into mature neuronal cells and is required to stimulate the differentiation of OPCs into mature oligodendrocytes [21-23]. As RA is able to act both on the immune response and to stimulate progenitor cell differentiation, we postulate that RA would be an ideal candidate to tackle the two pillars of MS, in this case, both the pro-inflammatory microenvironment due to the targeted destruction of myelin sheaths by activated immune cells, and the lack of remyelination of lesions sites in MS. As RA suffers from premature degradation and chemical instability, LNCs could be a prime delivery platform to allow RA to reach the CNS in therapeutically ideal concentrations to fully harness its therapeutic potential. To ensure that the LNC preferentially recognize the TfR expressed primarily at the BBB interface, the formulations were co-loaded with super paramagnetic iron-oxide nanoparticles (SPIONs) to promote magnetic guidance of the LNC towards the CNS by the presence of an external magnetic field close to the head of the patient. Several different works have focused on the use of SPIONs, and other drugs encapsulated within one nanostructure to achieve magnetic targeting [24, 25]. The SPIONs are also commercially available and considered safe for administration and are externally stabilized by the presence of oleic acid to grant them the necessary hydrophobicity to ensure their encapsulation within the LNC.

Herein, TfRp functionalized SPION-LNC RA were developed to achieve high rates of brain permeability and to significantly improve the pharmacokinetic properties of RA. The goal of this work was to assess the developed functionalized LNC on their ability to cross the BBB, and to interact with both OPCs and microglia in order to promote the production of new myelin and to control the inflammatory environment, that is characteristic in MS. The impact on cell metabolic activity and potential biological interactions of the TfRp-SPION-LNC RA was evaluated in microglia, OPCs and endothelial cells. Then, TfRp-SPION-LNC RA were evaluated for their potential to stimulate OPCs differentiation, as well as the potential to control the inflammatory response in an lipopolysaccharide (LPS)-activated microglia model.

2. Materials and methods

2.1- Materials

Transferrin-receptor binding peptide (Thr-His-Arg-Pro-Pro-Met-Trp-Ser-Pro-Val-Trp-Pro-Cys) was purchased from GenScript (Piscataway, NJ, USA); crosslinker (NH2-PEG-Mal, PEG of 5 kDa) from Biochempeg (Watertown, MA, USA) Labrafac® was kindly provided by Gattefosse SA (Saint-Priest, France); Phospholipon® 80 H Lipoïd® was purchased from Lipoid Gmbh (Ludwigshafen, Germany); Kolliphor® HS 15 was gifted from BASF (Ludwigshafen, Germany); DiD solid; DiIC18(5) solid and phalloidin-alexa fluor 488 was bought from Alfagene (Lisboa, Portugal); iron oxide(II,III), magnetic nanoparticles were acquired from Laborspirit, lda (Lisboa, Portugal); retinoic acid, millex-HV Syringe Filter Unit, 0.45 µm, sodium chloride, sodium hydroxide, sulfanilamide, glycerin, Tween® 80, hydrocortisone, ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), basic fibroblast growth factor (bFGF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-(1-Naphthyl)ethylenediamine dihydrochloride, poly(L-lysine), rabbit anti-Olig2, mouse anti α-tubulin, alexa-fluor 488, 546 and 647, paraformaldehyde egtazic acid, magnesium chloride, piperazine-N,N′-bis(2-ethanesulfonic acid), resazurin, sodium nitrite and sodium azide were purchased from Sigma (St. Louis, MO, USA); Hoechst was acquired from Molecular Probes (Eugene, OR, USA); DMSO from VWR (West Chester, PA, USA); rat anti-myelin basic protein was bought from AbD serotec (Raleigh, NC, USA); endothelial basal medium 2 (EBM-2) and high glucose with UltraGlutamineTM Dulbecco's Modified Eagle medium (DMEM) from Lonza (Basel, Switzerland); for high-pressure liquid chromatography (HPLC) analysis, trifluoroacetic acid (TFA) purchased from Acros Organics (USA) and Acetonitrile HPLC Gradient Grade purchased from Fisher Scientific (UK) were used; Triton X-100 from Spi-Chem (West Chester, PA, USA); fetal bovine serum (FBS), penicillin-streptomycin, chemically defined lipid concentrate, rat tail collagen type I and acetic acid, from Gibco (Waltham, MA, USA); ethanol from Valente e Ribeiro, Lda (Lisbon, Portugal); IL-6, Il-10 and TNF-α complete ABTS development kits were purchased from Peprotech (Rocky Hill, NJ, USA); E.coli O111:B4 LPS was acquired from Invivogen (San Diego, CA, USA); Hank's Balanced Salt Solution (HBSS) without calcium or magnesium supplemented with trypsin (0.0025% w/v) and 0.001mg.mL-1 DNAse I was purchased from Applichem GmbH (Darmstadt, Germany); normal donkey serum was acquired from Biosource (San Diego, CA, USA) Ultrapure water was prepared in-house.

Immortalized human cerebral microvascular endothelial cell line (hCMEC/D3 cell line) was purchased from Cedarlane (Hornby, ON, Canada), immortalized rat microglia cell line (BV-2) was kindly provided by Anne des Rieux, PhD from Université Catholique de Louvain, Belgium.

2.2- Lipid nanocapsules manufacture

LNC were prepared following the protocol previously developed by Heurtault et al. [10]. In brief, Kolliphor® HS 15 (0.450 g), Phospholipon® 80 H (0.045 g), NaCl (0.060 g), Labrafac® (0.600 g) and water (1.975g) were mixed with gentle magnetic stirring at 40 ºC for 10 minutes. Afterwards, the solution was slowly heated until 90ºC and cooled to 60 ºC a total of three times. During the last cycle, a rapid shock with cold water (10 g, at 4ºC) was added at 75-77 ºC under high-speed stirring. For RA loaded LNC, 250 µL of previously dissolved RA in DMSO (10 $mg.mL^{-1}$) was added at 79-81ºC at the last heating/cooling cycle. For SPIONs addition, 100 µL of a stock solution at 5 $mg.mL^{-1}$ in toluene was added simultaneously with RA. For blank LNC, 250 µL of water was added instead. Afterwards, the LNC were centrifuged at 3200 x g to remove all the unreacted lipids and then filtered through a 0.45 µm syringe filter. This process was replicated with a 10-fold increase in excipients as a pilot scale-up study (LNC RA 10x). For LNC DiD, 0.3 mg of DiD ($1mg.mL^{-1}$ in absolute ethanol) was previously dissolved in Labrafac® before the excipients were mixed, and then the same process mimicked. All formulations were then stored at 4ºC until further use.

2.2.1- Surface functionalization of lipid nanocapsules with a transferrin-receptor binding peptide

Transferrin-receptor binding peptide LNC (TfRp-LNC) were modified post-manufacturing by a two-step process. Firstly, NH2-PEG-maleimide was introduced on to the shell of the LNC via a transacylation reaction with Kolliphor® HS 15 [26]. 1 µM NH2-PEG-maleimide was added to 2 mL of LNC (molar ratio malameide:Kolliphor 1:90), followed by 10 mL of NaOH 10 M. This reaction took place at 25ºC for 15 minutes and was then stopped by an acidic glycine buffer. The NH2 reacts with the hydroxystearic acid present on Kolliphor® HS 15 and creates an amide bond with the LNC [27]. The unreacted NH2-PEG-malameide was separated by dialysis for 48

hours (dialysis membrane MWCO 10 kDa), with water changes at 2, 4, 24 and 48 hours. The second step consisted of the reaction of the maleimide with the terminal cysteine of the transferrin-receptor binding peptide (TfRp) (Thr-His-Arg-Pro-Pro-Met-Trp-Ser-Pro-Val-Trp-Pro-Cys). 500 µL of 0.5 $mg.mL^{-1}$ TfRp was added to the LNC (molar ratio 1:2.39) and kept under stirring at 150 rpm for 2 hours, vortexing every 30 minutes. Afterwards, 2 mM of NaCl and 0.1 mM of Cysteine hydrochloride were added to block all the unreacted maleimide at the surface of the LNC. The solution was then purified from unattached TfRp by dialysis for 48 hours with water changes at 2, 4, 24 and 48 hours.

2.3- Physico-chemical characterization of lipid nanocapsules

The physico-chemical properties of the LNC were assessed using a Malvern Zetasizer Nano ZS (Malvern Instruments). To measure the average size and polydispersibility index (PdI), the samples were diluted in 1/100 in deionized water. To evaluate the zeta-potential, the samples were instead diluted at the same concentration in NaCl 10 mM. These analyses were repeated at 3, 6 and 9 months to assess long-term stability of the nanosystem.

Transmission electron microscopy (TEM) characterization of the LNC was performed on using a JEOL JEM-1400 microscope. Samples were prepared by adding 10 µL of the suspension of the LNC onto a 300-mesh nickel grid and stained with 1% uranyl acetate.

Scanning electron microscopy (SEM)/EDS exam was performed using a High-resolution Scanning Electron Microscope with X-Ray Microanalysis and CryoSEM: JEOL JSM 6301F/ Oxford INCA Energy 350/ Gatan Alto 2500. The samples were rapidly cooled (plunging it into sub-cooled nitrogen – slush nitrogen) and transferred under vacuum to the cold stage of the preparation chamber. Afterwards, samples were fractured, sublimated (‘etched’) for 180 sec. at -90°C, and coated with Au/Pd by sputtering for 90 sec. SEM/EDS evaluations were carried out at a temperature of -150°C.

The association efficiency of RA was measured by the direct method. The LNC were destroyed by dissolution in methanol (1/20) to release the encapsulated RA. RA was then quantified by Reverse-phase High-performance Liquid Chromatography using a HPLC column-Xterra (3.5 µm) RP18. The mobile phase was composed of water and acetonitrile solution with 0.01%

Trifluoroacetic acid (TFA) in a 15:85 ratio. The method was run in an isocratic mode with a flow rate of 1mL/min and an injection volume of 20 µL. The encapsulation efficiency was calculated with the following equation:

$$AE\% = \frac{Measured\ retinoic\ acid}{Total\ added\ retinoic\ acid} \times 100$$

The total amount of SPIONs was assessed by the ortho-phenanthroline method [28], and was calculated by the following equation:

$$AE\% = \frac{Measured\ SPIONs}{Total\ added\ SPIONs} \times 100$$

2.4- Cell culture

2.4.1 Immortalized endothelial human cell culture (hCMEC/D3)

Immortalized human cerebral microvascular endothelial cells (hCMEC/D3) were grown in tissue cultured flasks, in EBM-2 supplemented medium with heat-inactivated FBS (5 %, v/v), penicillin-streptomycin (1 %, v/v), hydrocortisone (1.4 µM), ascorbic acid (5 µg.mL^{-1}), chemically defined lipid concentrate (1/100, v/v), HEPES (10 mM) and bFGF (1 ng.mL^{-1}). The bFGF was always added extemporaneously in the cell culture medium. Cells were sub-cultured every 5–6 days using trypsin–EDTA to detach them from the flasks. The culture medium was replaced every other day. Cells between the passages 48-58 were used.

2.4.2 Immortalized microglia rat cell culture (BV-2)

Immortalized rat microglia cell was grown in Dulbecco's modified eagle medium (DMEM) supplemented with FBS (5 %, v/v), penicillin-streptomycin (1 %, v/v) Cells were sub-cultured every 3–4 days using trypsin–EDTA to detach them from the flasks. The culture medium was replaced every other day. Cells between the passage 25-36 were used.

2.4.3 Primary rat oligodendrocyte precursor cell culture (OPCs)

All procedures involving animals and their care were performed in agreement with institutional ethical guidelines, the EU directive (2010/63/EU) and Portuguese law (DL 113/2013). Experiments described here had the approval of Portuguese Veterinary Authorities. Animals had free access to food and water, being kept under a 12-h light/12-h dark cycle.

Primary cultures of OPCs were obtained as previously described [29, 30]. Briefly, mixed glial cultures were isolated from the brains of P0-P2 Wistar Han rats. Isolated cortices were digested in Hank's Balanced Salt Solution (HBSS) without calcium or magnesium supplemented with trypsin (0.0025% w/v) and 0.001 $mg.mL^{-1}$ DNAse I for 15 minutes at 37ºC. Dissociated cortices were cultured in poly(L-lysine) (PLL) coated 75 cm2 flasks and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated (30 min, 56ºC) FBS and 1% (v/v) penicillin-streptomycin. When confluence was reached (~12 days) the flasks were shaken overnight on an orbital shaker (220 rpm) at 37ºC to remove loosely attached microglia and OPCs. OPCs were further purified by a differential selective adhesion step by seeding cells in suspension into non-tissue culture treated petri dishes for 2 hours to remove microglial cells. OPCs were then seeded on PLL pre-coated glass coverslips and maintained for 18 hours in OL SATO medium which was composed by DMEM medium supplemented with transferrin (0,1 $mg.mL^{-1}$), bovine serum albumin (BSA) (0.1 $mg.mL^{-1}$), putrescin (16 $\mu g.mL^{-1}$), progesterone (60 $ng.mL^{-1}$), thyroxine (40 $ng.mL^{-1}$), sodium selenite (40 $ng.mL^{-1}$), triiodo-L-thyroxine (30 $ng.mL^{-1}$), insulin (5 $\mu g.mL^{-1}$) and 0.5% heat-inactivated (30 min, 56ºC) FBS. After 18 hours, the media was discarded and replaced by OL SATO with LNC, free RA, TfRp-LNC RA and TfRp-SPION-LNC RA. After 4 hours, the media was discarded again and replaced by fresh OL SATO. Microglia and a small percentage of astrocytes were present in the cultures but did not influence the normal behaviour and differentiation of OPCs. Cells were evaluated at day 2 and 5 of differentiation.

2.5- Evaluation of the effect of lipid nanocapsules on cell metabolic activity

Cellular metabolic activity was assessed either through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (microglia and endothelial cells) or the resazurin assay (OPCs). Cells were seeded in a 96-well plate at a density of 6.25×10^4 viable cells/cm^2 for hCMEC/D3 and 3.13×10^4 viable cells/cm^2 for BV-2. The cells were then incubated for 48 hours (hCMEC/D3) and 24 hours (BV-2) and then the medium was removed, and the cells were washed twice with phosphate buffered saline (PBS). Afterwards, the cells were incubated with different concentrations of blank-LNC, free drug and TfRp-LNC RA, for 4 and 24 hours Afterwards, hCMEC/D3 and BV-2 cells were incubated with MTT solution (0.5 mg.mL^{-1} in medium) for 3 hours. Lastly, the MTT was removed and DMSO was added to dissolve the formazan crystals formed after a gentle room temperature (RT) shake. The absorbance was measured at 590 and 630 nm. The metabolic activity was calculated as a percentage compared to the cells incubated only with plain medium (EBM-2 and DMEM, respectively) and incubated with triton X-100 (1% v/v, in PBS). Data was referenced to a 70% cell viability threshold according to ISO 10993–5 [31].

OPC metabolic activity was inferred through the resazurin methodology at day 2 and 5 of differentiation. The cells were incubated with 10% (v/v) solution of resazurin diluted in OL SATO and cells for 3 hours. Afterwards, 100 µL was transferred to a dark 96-well plate Cell metabolism was inferred by measuring the levels of produced resofurin, which is a molecule produced by the reduction of resazurin (redox-sensitive fluorescent dye) [32]. Fluorescence was measured at an excitation of 530 nm and emission of 590 nm using a fluorometer Synergy Mx (BioTeK Instruments, GenX software).

2.6- Evaluation of lipid nanocapsules-cell interaction

The ability by the target cells to interact with and internalize the LNC is of paramount importance to ensure the biological activity of RA. To quantify the internalization by endothelial cells of the BBB, hCMEC/D3 were seeded in a 6-well plate at a density of 6.25×10^4 viable cells/cm^2 and

were left to adhere overnight. The cells were then washed twice with PBS and then incubated with TfRp-SPION-LNC DiD, LNC DiD and TfRp-SPION-LNC DiD + TfRp for 1 hour and 4 hours (data not shown). After the incubation, the cells were washed twice and detached by trypsin-EDTA. The cells were then fixed with PFA 4% in PBS for 20 minutes (RT), washed twice with PBS and resuspended in PBS filtered through a 70 µm mesh and placed in cytometer tubes. The fluorescence intensity was analyzed with a flow cytometer (Accuri).

To obtain a qualitative analysis of the interaction with hCMEC/D3 cells, they were seeded in glass cover slips in a 24-well plate at a density of 3.1×10^5 viable cells/cm^2. After 48 hours in culture, the medium was removed and the cells were washed twice with pre-warmed PBS, and then incubated with TfRp-LNC DiD or LNC DiD. Additionally, cells were pre-treated with TfRp before incubation with TfRp-LNC DiD to evaluate whether targeted-LNC uptake is specific of TfR. The equivalent dye concentration encapsulated within the LNC was set to 1 µg.mL^{-1}. After 1 hour incubation (37ºC, 5% CO2), the cells were washed and fixed with 4% PFA. Afterwards, the cells were stained with Alexa Fluor 488 nm phalloidin and hoescht (405 nm) and mounted with fluoromount mounting media. Afterwards the cells were observed with a laser scanning confocal microscope (Leica SP5, 405 nm for Hoescht, 520 nm for phalloidin and 644 nm for DiD).

For BV-2, the cells were seeded at a density of 1×10^5 viable cells/cm^2, and the same protocol from above was applied. After 4 hours of incubation the cells were washed again and fixed with 4% PFA and permeabilized and blocked in PBS containing 5% (v/v) normal donkey serum (NDS) and 0.3% (v/v) Triton X-100. The primary antibodies were diluted in PBS and incubated overnight in a humid chamber at 4ºC. The following primary antibodies were used: rabbit anti-Iba-1 (1:500), mouse anti-alpha tubulin (1:2000). The following day, secondary antibodies Alexa-Fluor 488 and 568 (1:1000) were applied for 1h at RT and subsequently treated for nuclear counterstaining at RT with Hoechst at 2 µL.mL^{-1}, and subsequently mounted with fluoromount mounting media. The cells were then observed by scanning confocal microscope (Leica SP5, 405 nm for Hoescht, 488 for Iba-1 and 568 for tubulin.

The OPCs were seeded on glass coverslips in a 24-well plate at a density of $3.1x10^4$ viable cells/cm^2. After 24 hours for cellular adhesion, the medium was removed, and the cells were treated with TfRp-SPION-LNC DiD at an equivalent encapsulated dye concentration of 0.1 µg.mL^{-1}. After 4 hours of incubation, the medium with the LNC was removed and fresh medium

was added. At day 2 and 5 of differentiation, the cells were fixed with 4 % (v/v) paraformaldehyde containing microtubule stabilizers [29] (MP-PFA, 4% PFA with the addition of 65 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 25 mM of HEPES, 10 mM of egtazic acid (EGTA) and 3 mM MgCl2, for 15 min and further permeabilized and blocked in PBS containing 5% (v/v) NDS and 0.3% (v/v) Triton X-100. Primary antibodies were diluted in PBS containing 1% (v/v) NDS and 0.15% (v/v) Triton X-100 and incubated overnight in a humid chamber at 4ºC. The following primary antibodies were used: rabbit anti-Olig2 (1:400), rat anti-MBP (1:100). Secondary antibodies Alexa-Fluor 488 and 568 were applied for 1h at RT and subsequently treated for nuclear counterstaining at RT with Hoechst at 2 $\mu L.mL^{-1}$. Afterwards, the cells were mounted using FluoromountTM mounting media. The cells were then observed with scanning confocal microscope (Leica SP5, 405 nm for Hoescht, 488 nm for Olig2, 568 nm for myelin basic protein (MBP) and 644 nm for DiD). Cell images were analyzed using Fiji ImageJ software version 2.3.0.

2.7 - Permeability assays in a blood-brain barrier model

A BBB in vitro model, based on a transwell system, was adapted from a previous work [16] to evaluate the permeability of the LNC nanoparticles. Briefly, 2.5 x 10^4 viable cells/cm^2 of hCMEC/D3 cells (500 µL of medium) were seeded in 12-well plates in transwell® cell culture inserts with a 1 µm pore size (apical side). Before the seeding, the inserts were coated with 90 µL of a 50 $\mu g.mL^{-1}$ rat tail collagen type I previously diluted in an acetic acid solution (0.02 M) for 1 h (37°C, 5% CO2). Afterwards, inserts were washed twice with PBS pH 7.4. The basolateral side was filled with 1.5 mL of medium. The system was maintained in the incubator at 37 ºC with 5 % CO2 for 8 days, and medium was changed every 2 days. The hCMEC/D3 cells monolayer became confluent at the day 8 after seeding, which was the day appropriate to perform the permeability study. The integrity of the cell monolayer was checked every other day by monitoring the trans endothelial electric resistance (TEER) using an endothelial Volt–Ohm meter (Millicell® ER S-2; Merck Millipore, Billerica, MA, USA). The resistance value of an empty filter with only media was subtracted from each measurement.

The permeability experiment was performed in the apical-to-basolateral direction. After removing the cell culture medium, the basolateral compartment was filled with 1.5 mL of HBSS, ensuring sink conditions. Regarding the apical compartment, 500 µL of RA at 0.1 and 1 µg.mL^{-1}, in the free form or loaded on either TfRp-SPION-LNC or non-functionalized LNC, diluted in HBSS were added. Then, the assay was conducted at 37 ºC using an orbital shaker incubator (100 rpm). At different time points (60, 120, 180 and 240 min), 200 µL samples were taken from the basolateral side, and the same volume of pre-heated HBSS was added to replace the withdrawn volume. At the last timepoint, the monolayer was disrupted with the presence of 1% Tween-80 in order to lyse the cells to quantify intracellular RA. The integrity of the cell monolayer was checked during the permeability experiment by monitoring the TEER. The apparent permeability coefficient, Papp (cm.s^{-1}), measures drug permeability across the cell monolayer, and is calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

where dQ (µg) is the amount of drug that permeated from the apical to the basolateral compartment during the incubation time t (s), A is the surface area of the transwell® insert (cm2) and C0 the initial concentration of the drug on the apical side (µg.mL^{-1}). The samples were analyzed by the method described above for HPLC, with some adaptations proposed by us. 20 µL of each sample was separated on an HPLC Vanquish (Thermo Fischer Scientific, Bremen, Germany). The column was a Accucore RP-MS column (Thermo Fischer Scientific, Bremen, Germany) 2.6 µm particle size and dimensions 100 mm ID x 2.1 mm. The samples were eluted in isocratic conditions with 15% solvent A (miliQ water with 0.1% v/v formic acid) and 85 % of solvent B (acetonitrile with 0.1% v/v formic acid) during 15 min at a flow rate of 0.350 mL/min.

The Analysis was done on an Orbitrap Exploris 120 mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by Orbitrap Exploris Tune Application 2.0.185.35 and Xcalibur 4.4.16.14. The capillary voltage of the electrospray ionization source (ESI) was set to 3.5 kV. The capillary temperature was 300ºC. The sheath gas, auxiliary and sweep gas flow rate were at 50, 10 and 1 (arbitrary unit as provided by the software settings). Single ion monitoring (SIM) was used to detect RA (300.2084 Da). The resolution of MS scan was 60 000.

Data dependent MS/MS was performed on HCD using nitrogen as gas with collision energy settings of 30 V.

MS data handling software (Xcalibur QualBrowser software, Thermo Fischer Scientific) was used to search extrapolate the concentration of RA by their m/z value and MS/MS value.

2.8- Anti-inflammatory action of lipid nanocapsules

To investigate if TfRp-SPION-LNC RA were able to adequately suppress the microglia activation that occurs in the CNS in demyelinating disorders, BV-2 were pre-emptively activated with LPS, as an inflammatory trigger. Briefly, BV-2 cells were seeded in 24-well plates at a density of 1×10^5 viable cells/cm^2 and left to adhere for 24 hours. After 24 hours, the medium was removed, and the cells were washed twice with PBS followed by the addition of 100 $ng.mL^{-1}$ of LPS for 1 hour. Microglia cells were then washed again and the free drug, blank-LNC, TfRp-LNC RA and TfRp-SPION-LNC RA were added in different concentrations to the cells and incubated for 4 hours. After 4 hours, the supernatant of the cells was collected and analysed by ELISA for the presence of interleukin-6 (IL-6), interleukin-10 (IL-10) and tumour necrosis factor-alpha (TNF-α).

2.9 – Effect of lipid nanocapsules on the production of intracellular nitric oxide

Nitrites, stable metabolites of nitric oxide (NO), were assessed in the microglial cell line BV-2 after 1 hour of LPS stimulation to verify the potential of the LNC to revert the production of NO. Nitrites were dosed using the Griess assay as previously described. Briefly, cells were plated in a 24-multiwell plate at density of 2.5×10^4 viable cells/cm^2 and were exposed to LPS at concentration of 100 $ng.mL^{-1}$ for 1 hour. Afterwards, cells were incubated with blank-LNC, RA, TfRp-LNC RA and TfRp-SPION-LNC RA for another 4 hours. Lastly, the supernatants were incubated into a 96-well plate with an equal volume of Griess reagent. The absorbance was measured at a wavelength of 550 nm after 1 hour of incubation at RT using a fluorescent microplate reader (BioTeK® Synergy MX, Winnoski, VT, USA).

2.10 – Oligodendrocyte-progenitor cell immunocytochemistry and image acquisition

OPCs were seeded in a 24-well plate at a density of $3.1x10^4$ viable cells/cm^2. After 24 hours for cellular adhesion, the medium was removed and the cells were incubated with LNC, RA, TfRp-LNC RA and TfRp-SPION-LNC RA. After 4 hours, the medium with the LNC was removed again and fresh medium was added. At day 2 and 5 of differentiation, the cells were fixed following the same procedure described in 2.6, for 15 min and further permeabilized and blocked in PBS containing 5% (v/v) normal donkey serum (NDS) and 0.3% (v/v) Triton X-100. Primary antibodies were diluted in PBS containing 1% (v/v) NDS and 0.15% (v/v) Triton X-100 and incubated overnight in a humid chamber at 4ºC. The following primary antibodies were used: rabbit anti-Olig2 (1:400), rat anti-MBP (1:100) and mouse anti-alpha tubulin (1:2000). Secondary antibodies Alexa-Fluor 488, 568 and 647 were applied for 1h at RT and subsequently treated for nuclear counterstaining at RT with Hoechst at 2 $\mu L.mL^{-1}$. Qualitative images of the OPCs were obtained using a laser scanning confocal microscope (Leica SP5, 405 nm for Hoescht, 488 nm for Olig2, 568 nm for MBP and 647 nm for tubulin).

OPCs were imaged using the automated widefield microscope INCell Analyzer 2000 (Cytiva), with a 20x objective (N.A 0.45). 49 equally spaced fields were acquired in a 2D mode with 2x2 binning. Images were segmented using ilastik (version 1.3.3, https://www.ilastik. org/), followed by further segmentation and analysis using CellProfiler (version 3.1.9, https://cellprofiler.org). Final results were analyzed using Microsoft Excel (Microsoft Office 365). Outputs include the number of MBP and Olig2 positive cells, the area occupied by MBP, and the differentiation stages of oligodendrocytes based on tubulin markers. For the oligodendrocyte differentiation stages classification a method developed by Bouçanova et al. was adapted [33]. Briefly, an area around the nucleus was created and termed “collar”. Only Olig2 and well-segmented cells (area of nucleus defined between 60 and 192 μm2 and area of the collar defined between 720 and 1000 μm2) were considered for the analysis. The area of the tubulin protrusions was measured inside the collar area and the percentage of occupancy was related with the oligodendrocyte stage. Cells in stage I were classified as having less than 20% of occupancy area, in stage II between 21 and 32%, stage II between 33 and 51% and finally, stage IV cells more than 52%.

For the analysis of the MBP and the Olig2 positive cells, classification was performed using CellProfiler Analyst (version 2.2.1, https://cellprofiler.org/cp-analyst/), through the Random Forest algorithm, with user-defined classes. At least 100 objects were included in each class, to create a complete training set and to ensure reliable outcomes. Once the accuracy of classification was above 90%, the classifier was used for batch processing and the cells were staged using Microsoft Excel.

2.11 - Statistical analysis

For the performed statistical and data analysis, the software GraphPad Prism Version 9.0.1 was used. To verify the differences between groups, one-way or two-way analysis of variance (ANOVA) were applied. Normality was verified by the Shapiro-Wilk and Kolmogorov-Smirnov test. In case of no normality, a logarithmic transformation was applied, and the results analysed by one-way ANOVA. Differences between groups were compared with a post hoc test (Tukey's honestly significant difference). Results are reported as mean ± standard deviation (SD) from a minimum of three independent experiments. Differences were considered significant at *$p<0.05$, **$p<0.01$, ***$p<0.001$ or ****$p<0.0001$.

3. Results and Discussion

3.1- TfRp-SPION-LNC RA production and characterization

TfRp-SPION-LNC RA were proposed to improve the targeting potential to the brain and to maximize the therapeutic effectiveness of compounds towards the CNS in demyelinating conditions. The SPION-LNC RA were developed by a phase-inversion methodology as previously described [10]. To promote the covalent link between the cysteine amino acid of the TfR-binding peptide and the surface of the LNCs, the shell of the LNCs was modified to present a functional group - maleimide was chosen because of the ease of rection with the thiol entity of the cysteine amino acid of the TfR-binding peptide [34].

The physico-chemical characterization of the TfRp-LNCs showed that the developed nanosystem presented an average size of around 100 nm, a monodisperse size distribution (average PdI of around 0.2), and a slightly negative zeta-potential. To achieve this size, the ratio of the excipients respected a previously determined balance [35]. Previous studies have showed that particles of around 100 nm have an improved interaction with the targeted receptors at the surface of BBB cells, and even in some cases passive diffusion through the BBB tight junctions [36-38]. Across all the formulations, high encapsulation (over 88%) of RA was always accomplished due to the innate affinity of RA to the oily lipids present at the core of the LNC. Interestingly, when the process was scaled-up, the association efficiency was even higher, with practically all RA being encapsulated. This is likely due to the higher quantities of available lipids to react with the RA ensuring a more efficient encapsulation process.

The modification of the LNCs with the TfR-binding peptide resulted in a significant increase in the size of the LNCs (from 77 nm to 109 nm, respectively) and made them more heterogenous regarding size distribution (0.134 to 0.299 PdI, respectively). This phenomenon was already reported as a consequence of the process of surface functionalization per se [39]. Despite this, the TfRp functionalization did not affect the preservation of the payload of the LNCs. The functionalization process was verified by the presence of elemental Sulphur on the final formulation compared to the absence of it in unfunctionalized batches by elemental dispersive scanning (Table S1). Likewise, the presence of SPIONs did not impact the size and PdI and generate minor variations regarding the zeta potential. This could be due to some SPIONs depositing around the shell of the LNC, as complete encapsulation was not confirmed, since it was not feasible to separate the destroyed LNC from the released SPIONs. Additionally, when using the ortho-phenanthroline, it should be noted that only elemental iron is quantified, and some of the iron from SPIONs might not be fully reduced from magnetite.

The characteristics of the different formulations are presented in Table 1.

Table 1. - Physico-chemical properties of the different formulations of RA-loaded and unloaded LNC. Results from n=3 independent experiments, mean +/- SD. *p<0.05, compared to the blank-LNC.

Formulation	Size (nm)	PdI	Zeta-potential (mV)	RA association efficiency (%)	SPION association efficiency (%)
Blank-LNC	77 ± 1	0.134 ± 0.006	-3.51 ± 0.67	-	-
LNC RA	70 ± 3	0.130 ± 0.020	-3.69 ± 1.01	90.6 ± 3.1	-
SPION-LNC RA	78 ± 1	0.126 ± 0.018	-8.34 ± 1.65	87.6 ± 2.8	53.5 ± 0.5
TfRp-SPION-LNC RA	109 ± 12*	0.299 ± 0.034	-7.21 ± 0.80	87.6 ± 2.8	53.5 ± 0.5
LNC RA (10x)	68 ± 1	0.081 ± 0.017	-5.94 ± 0.58	98.4 ± 0.5	-
SPION-LNC DiD	74 ± 0	0.200 ± 0.010	-2.73 ± 0.20	-	53.5 ± 0.5

TEM and cryo-SEM imaging were performed to observe the LNC morphology, and to understand their overall organization and structure (Figure 1). The images showed a size-uniform population that is in accordance with the observed PdI (Table 1). Due to the small size of the LNC, the cryo-SEM images appeared to have some mild deformation. This is due to the intensity of the electron beam on the LNC due to their smaller size, bordering on the resolution limit of the cryo-SEM.

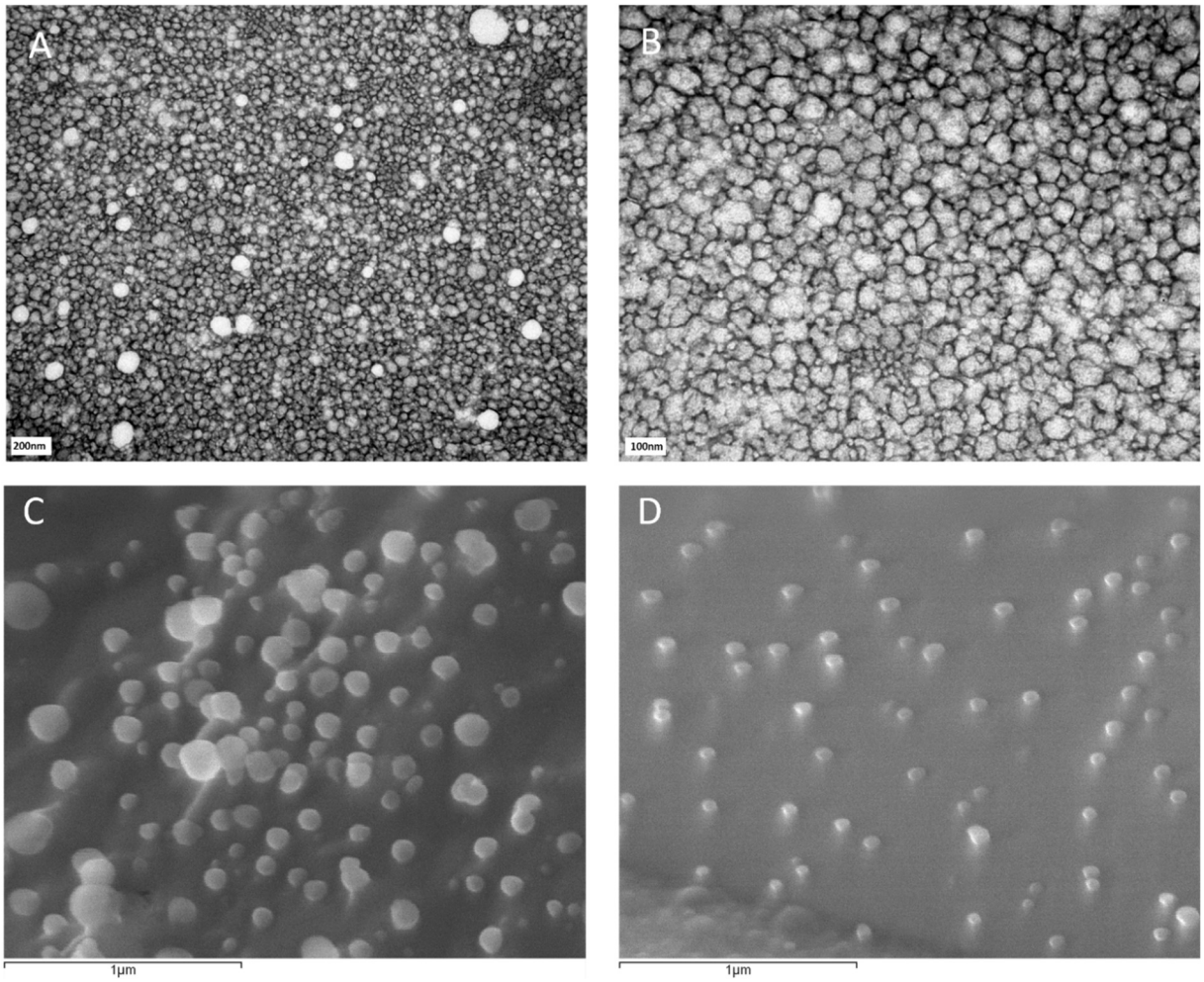

Figure 1 – TEM (Top) and cryo-SEM (bottom) of SPION-LNC RA. The SPIONs were not observable under TEM due to their minute size (5 nm). (A) 50000x magnification, scale bar corresponding to 200 nm, (B) 100000x magnification, scale bar corresponding to 100 nm (C) and (D) cryo-SEM of two different fields, scale bar corresponding to 1 µm.

To confirm that the LNC were able to preserve RA and were ideal candidates for long-term storage at 4º C, their physico-chemical properties were assessed every 3 months (Table S2). After 9 months of storage, the LNC were able to preserve their size around 70 nm, PdI of 0.1 and around 90% encapsulation efficiency of RA without premature degradation. This was verified only for unfunctionalized LNC, as the functionalization process was always done previous to any assay.

The release profile of RA from TfRp-SPION-LNC RA was also verified and compared with the dissolution profile of free RA. Through 196 hours, the release profile was found to be less than 1% in simulated biological media (Figure S1). This is in accordance with previously reported release profiles of LNC as they tend to have sustained release due to their innate stability, and only release their payload after fusion with the cell membranes [40].

Fluorescently labelled LNC encapsulating DiD instead of RA were developed to be used in assays were live fluorescent tracking of the LNC was required. Anthocyanin dyes have been used in lipidic nanosystems [41] due to their ease of encapsulation and overall stability without premature release of the system [42]. The physico-chemical properties of LNC incorporating DiD were identical to LNC encapsulating RA (Table 1), meaning that these are ideal candidates to mimic the properties of the properties of these in imaging assays.

3.2- In vitro nanocapsule safety and cell interaction

3.2.1 – Effect of lipid nanocapsules on cell metabolic activity

One of the main obstacles to the use of LNC for brain delivery is their innate cytotoxicity. Specifically, the high concentrations of lipids and surfactants at the surface of LNC make them noticeably toxic for cells, thus special considerations should be accounted for when designing LNC based nanosystems. As LNC normally hold high encapsulation efficiencies of lipid drugs, this issue can be avoided by promoting higher dilutions to reduce the lipid content to tolerable levels. Here the cytotoxicity of the developed LNC formulations was studied after 4h and 24h of exposition on hCMEC/D3 brain endothelial cells, BV-2 microglia cells and primary rat OPCs. The MTT and resazurin assays were chosen to infer the cytotoxicity profiles from cellular metabolic changes. A range of concentrations was chosen, that was selected based on the quantity of lipids of the LNC, and expressed in its equivalent concentration of RA for the blank-LNC, TfRp-LNC RA and the free RA. The concentrations were based on the range of concentrations wherein RA was shown to have an impact on the promotion of differentiation of neural stem cells of the CNS and suppress activated microglia [2, 43].

On hCMEC/D3 cells it was noticeable some toxicity at 4 hours for the highest concentrations, although still above the 70% threshold for all the LNC formulations (Figure 2). At 24 hours, the toxicity at higher concentrations is severe, even for free RA, but at the lowest concentration the LNC and RA were still well tolerated.

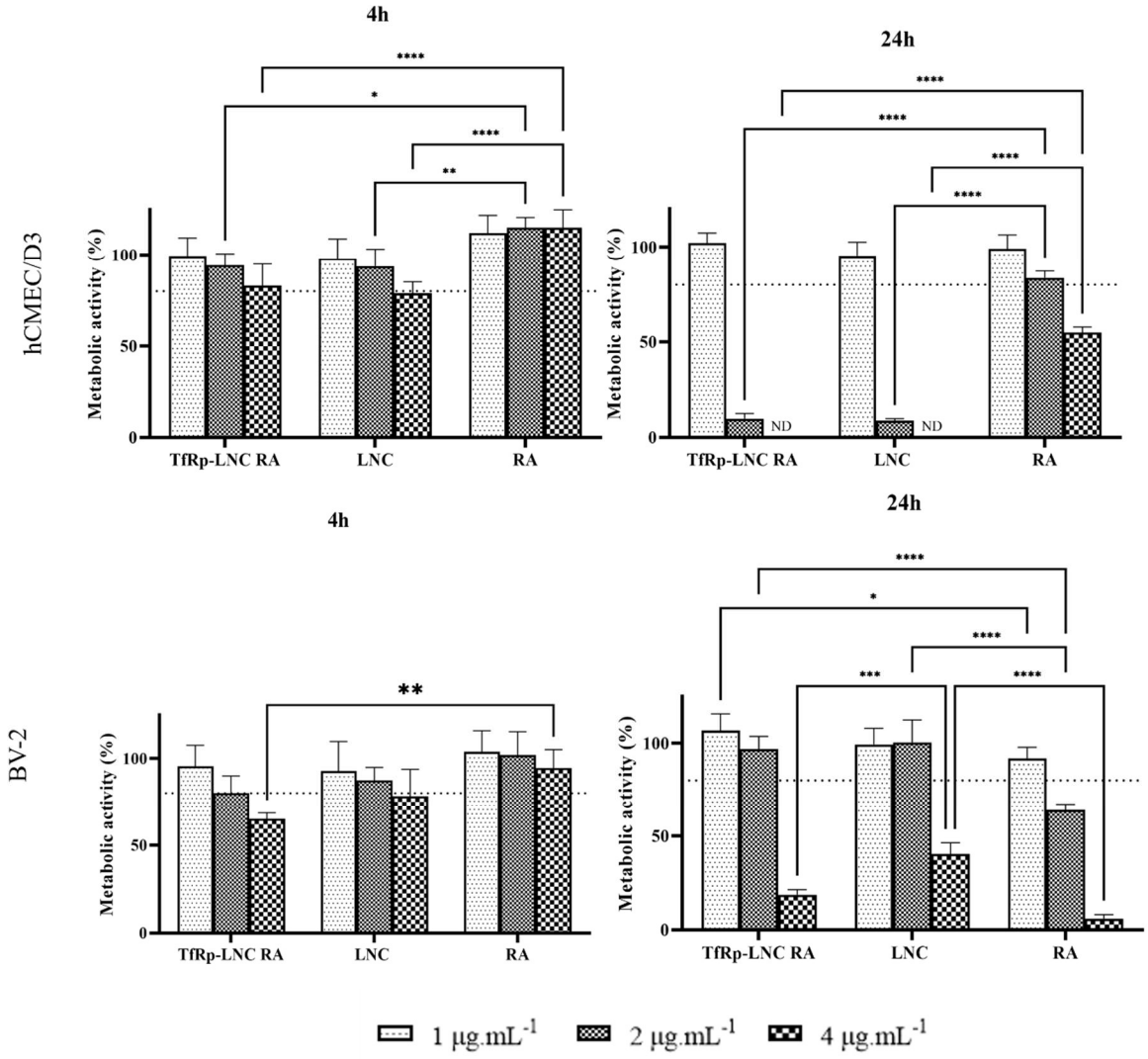

Figure 2 – Metabolic activity of brain endothelial cells (hCMEC/D3, top graphs) at 4 h and 24 h. Metabolic activity of microglia cells (BV-2, bottom graphs) at 4 h and 24 h. The concentration of RA was calculated based upon dilution factors of LNC. Results from n=3 independent experiments (three replicates per condition); Mean +/- SD *p<0.05; **p<0.01; ***p<0.005; ****p<0.0001.

It is worth noting that the results were similar to reported values in the literature. In one study, LNC decorated with cannabidiol were tested on hCMEC/D3 cells and it was found that, at an equivalent lipid concentration, the LNCs were tolerated above the 70% threshold [41]. It should be highlighted, however, that different excipient quantities were used between studies, thus, a straightforward comparison cannot be established. Regarding BV-2, the most notable result was the toxicity experienced at 4 h by LNC RA, that was not seen in the unloaded control. Herein, this could be due to a synergistic effect of the toxicity of the LNC and the RA at higher concentrations, as RA is known to suppress activated microglia [44]. At 24 h, similarly to hCMEC/D3, marked toxicity was observed at higher concentrations with LNC, but also significant toxicity of the higher concentrations of RA. Paradoxically, one study tested polymeric nanoparticles with RA at higher equivalent concentrations of RA, although on a different microglial cell line [45]. The study, however, did not show the marked toxicity exhibited in our results, perhaps symbolizing a potential different response of microglial cell lines to the presence of RA. With the results herein presented of cell metabolic activity assays, the concentration of 1 $\mu g.mL^{-1}$ was considered as the threshold for the following assays.

However, when the OPCs were assayed on the same concentrations at hCMEC/D3 and BV-2, it was found that at the higher timepoint (24 hours) and the same concentrations as the other cells, complete toxicity was observed (data not shown). Thus, as the primary cells were more sensitive to the action of the LNC, for any assay regarding the OPCs, lower concentrations and timepoints were used (Figure 3). The LNC were well tolerated at selected concentrations (0.01 $\mu g.mL^{-1}$ and 0.1 $\mu g.mL^{-1}$) and time point (4h of incubation). These results are in line with previously reported works in the literature, where LNC at equivalent surface lipid concentrations was incubated for 1 hour with OPC and showed no toxicity after 1 and 7 days [27]. Another work demonstrated that a nose-to-brain delivery route of LNC encapsulating the anti-inflammatory bioactive lipid prostaglandin D2-glycerol ester (PGD2-G) in an experimental autoimmune encephalomyelitis model had no impact on myelinating oligodendrocytes or OPC differentiation [46].

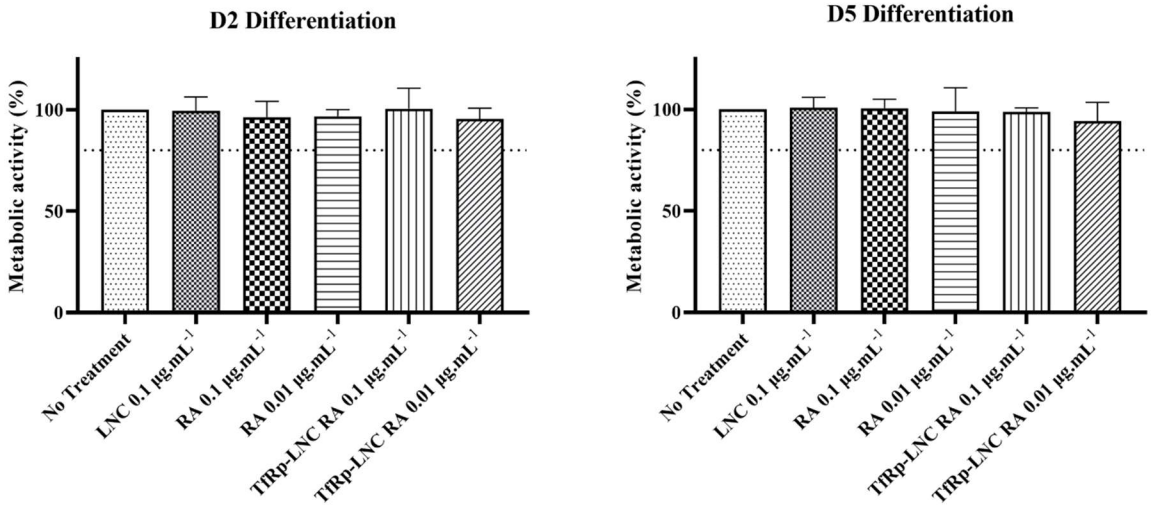

Figure 3 – Metabolic activity of primary OPCs in the presence of blank-LNC, free RA, TfRp-LNC RA. Values were normalized to the non-treated cells. Results from n=3 independent experiments (two replicates per condition); Mean +/- SD. *p<0.05.

3.2.2 – Assessment of lipid nanocapsule-cell interaction

The cell interaction of TfRp-SPION-LNC was performed using DiD as a fluorescent marker to track the cells and it was verified by flow cytometry. Confocal microscopy was also used to allow a qualitative analysis of the interaction of the LNC with the BBB endothelia. DiD was encapsulated within the matrix of the LNC. As the fluorescent dye DiD is described as stable within the LNC core, even in lipophilic environments, the observable fluorescence is indicated to be the TfRp-SPION-LNC DiD and not the released dye [42].

The outcomes of flow cytometry were expressed through the median fluorescence €ntensity (MFU), as represented in figure 4. It is observable that the presence of TfRp at the surface of LNC improved 3- to 4-fold the cell uptake when compared to the unfunctionalized LNC, suggesting the use of the TfR pathway to internalize the LNC. Furthermore, when establishing a direct competition assay, in the presence of TfRp previously added to the media, the uptake was slightly below the functionalized LNC, but still 2-fold higher than the unfunctionalized LNC,

suggesting the high affinity of the LNC to the TfR receptor. These results are in line with previously reported results regarding the functionalization of the surface of the LNC, which has already been demonstrated before as being significantly beneficial to their uptake by target cells despite the high rates of unspecific uptake due to the lipid composition of the LNCs [27, 31].

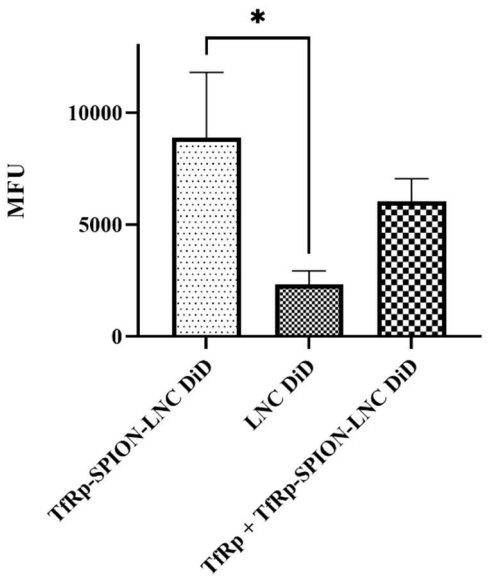

Figure 4 – Flow cytometry analysis of the in vitro cellular uptake of TfR-SPION-LNCs by hCMEC/D3 cells, expressed in mean fluorescence intensity (MFU). Results from n=3 independent experiments, Mean +/- SD, * $p < 0.05$.

To verify the results from flow cytometry, confocal microscopy analysis showed that the TfRp-SPION-LNC have an increased interaction with the cells, as compared to the unfunctionalized LNC. The localization of the internalized LNC, with a widespread distribution throughout the cell rather than membranal accumulation, which is more indicative of pinocytic uptake rather than endosomal accumulation suggests that the LNC were uptaken by receptor-mediated transcytosis[47]. To confirm that the LNC were in fact inside the cells and not deposited around the cell membrane, the orthogonal projection is presented. The results from the physico-chemical alterations, flow cytometry and confocal microscopy strongly suggest the successful

functionalization of TfRp on the surface of SPION-LNC RA. We attempted to quantify the association of the peptide with the LNC by nuclear magnetic resonance (NMR) but due to the low values of peptide and the thiol bond the resolution was not able to show the chemical bond (data not shown). To further verify if the interaction between the LNC and the cells was due to TfRp and not unspecific uptake, the functionalized LNC were incubated with cells that were pre-treated with TfRp, to demonstrate a competitive assay for the receptor target. As it is visible in Figure 5, the cells pre-treated with TfRp had noticeably less internalization than the functionalized cells without competition, suggesting that the uptake is due to the transferrin receptor. These results are in line with previous results at our group [18, 48], where poly(lactic-co-glycolic) acid (PLGA) nanoparticles were functionalized with TfRp, in order to target the BBB in CNS disorders, and an increase in the interaction between the nanoparticles and hCMEC/D3 was verified in both studies.

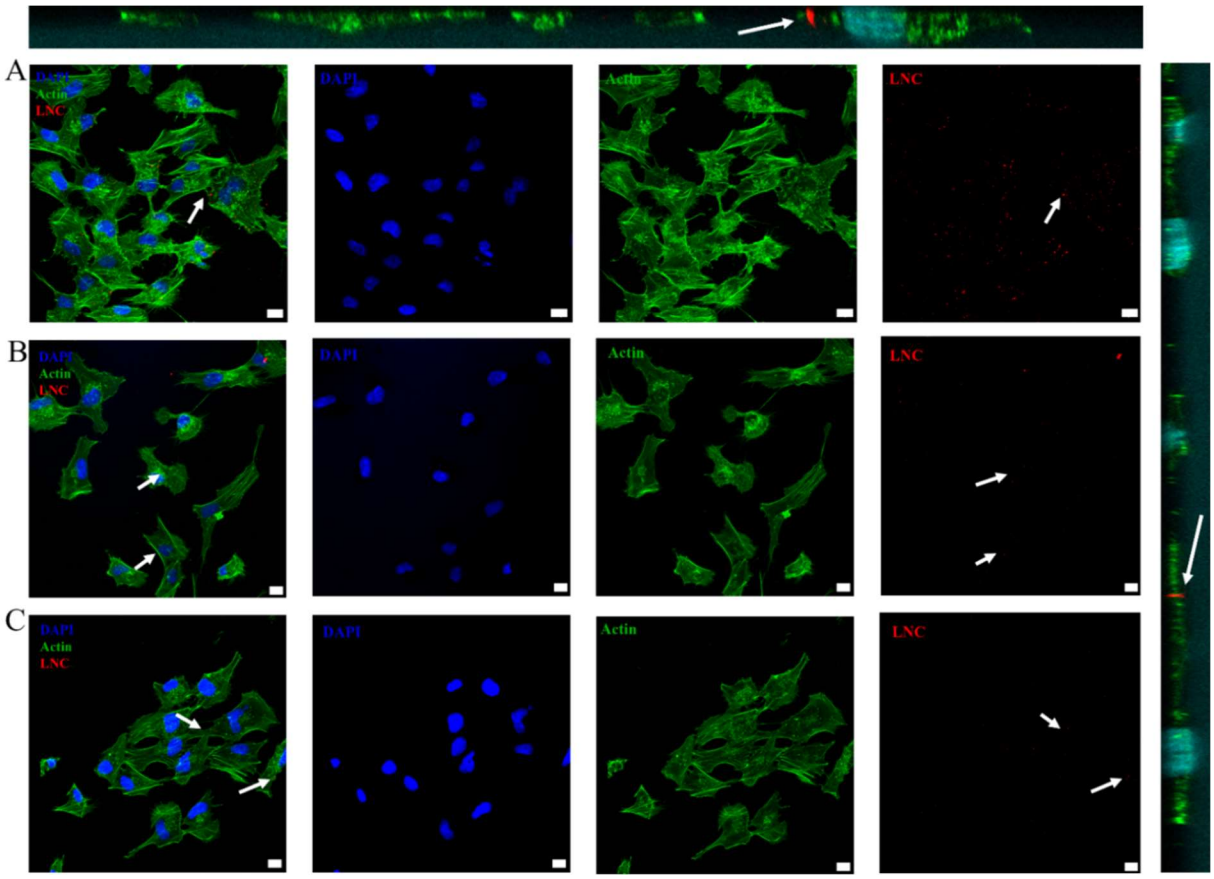

Figure 5 – Confocal fluorescence microscopy representative images of the internalization of the TfRp-SPION-LNC DiD by hCMEC/D3 cells after an incubation period of 1 hour. The internalization of the LNC was significantly higher on TfRp functionalized SPION-LNC DiD (A) compared to the unfunctionalized LNC (B) and the functionalized SPION-LNC DiD with TfRp pre-treatment as a competitor assay. Top side and right side are the orthogonal projection of TfRp-SPION-LNC DiD (A) showing the internalized LNC, the arrows indicate the signal from the LNC within the membrane of the cells. The localization of the TfRp-SPION-LNC DiD, both deep within the cells' cytoplasm and on the outskirts of the membrane is a good indicator of specific interaction with the cells. Scale bar = 10 μm.

The successful functionalization of a nanoparticle is critical to improve targeting to desired states and reduce the off-target effects of the drug delivery system. In this study, we have successfully demonstrated the attachment of a proven ligand that is able to guide LNCs to the BBB, which will allow controlled delivery of RA to the CNS. This is of extreme importance as the CNS is a tissue where RA was not able to reach in sufficient concentrations to have a biological effect due to poor pharmacokinetic properties.

Having this in mind, after the LNC can successfully manage the BBB, it is of paramount importance to verify their interaction with the two main target cells of the CNS within the scope of this study. As microglia are the main resident immune cells of the CNS and are usually present in neurodegenerative disorders at lesion sites in an activated site promoting the existence of an inflammatory environment. To promote the ideal conditions for the development and differentiation of OPCs into mature oligodendrocytes and result in new myelin being produce, reverting the microglia to a pro-resolutive state is a key step in ensuring the reversion of the myelin damage. Thus, it is required that the LNC can successfully interact and be internalized by microglia. The LNC demonstrated the ability to be internalized by the microglia (Figure 6) without triggering cell apoptosis or altering their conformation towards an aggressive, scavenging, activated state. The control without treatment was performed in order to validate the impact of the LNC on microglia (Figure S2)

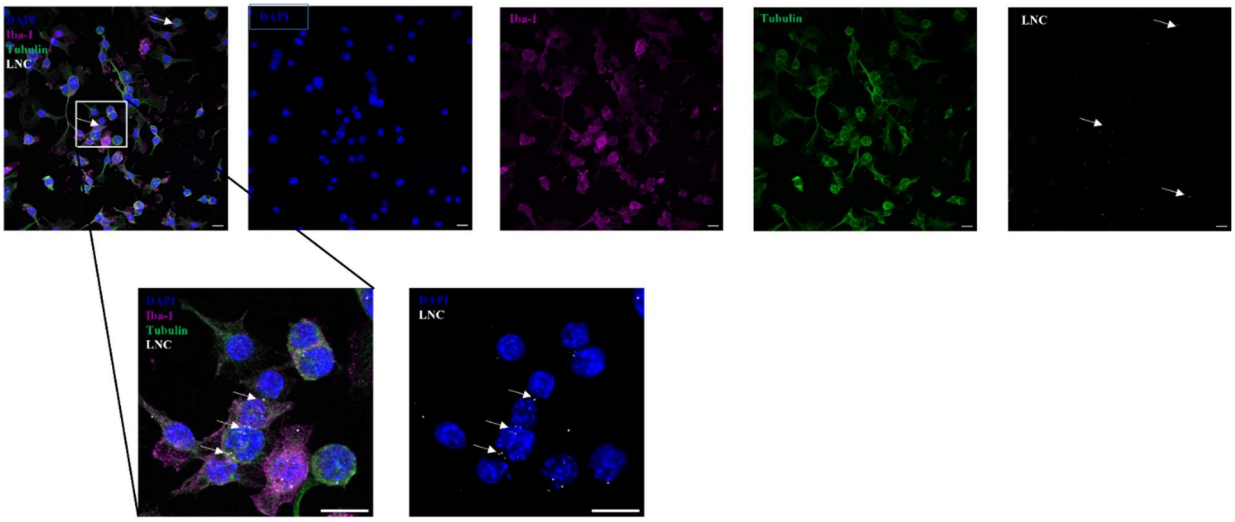

Figure 6 – Confocal fluorescence microscopy of the internalization TfRp-SPION-LNC DiD by microglia (BV-2) after an incubation period of 4 hours. The LNC was found to be located deep within the microglia, and to not promote their alteration towards an ameboid state, suggesting that it does not trigger microglial activation. The arrows indicate the presence of the TfRp-SPION-LNC DiD. Magenta: Iba-1, Green: Tubulin, Blue: Hoescht. Scale bar = 20 μm

The internalization of the TfRp-SPION-LNC DiD was verified at both days 2 and 5 of differentiation of OPCs, with the localization being deep within the cellular membrane of these cells (Figure 7). The presence of the LNC in both early stage and late stage OPCs, as observable between the differing OPCs in the days 2 and 5 of differentiation, can hint towards the ability to interact early in the maturation stage of OPCs to promote improved differentiation of the OPCs towards mature, myelinating oligodendrocytes. To verify if the LNC had any impact on the OPCs, controls without LNC were performed (Figure S3).

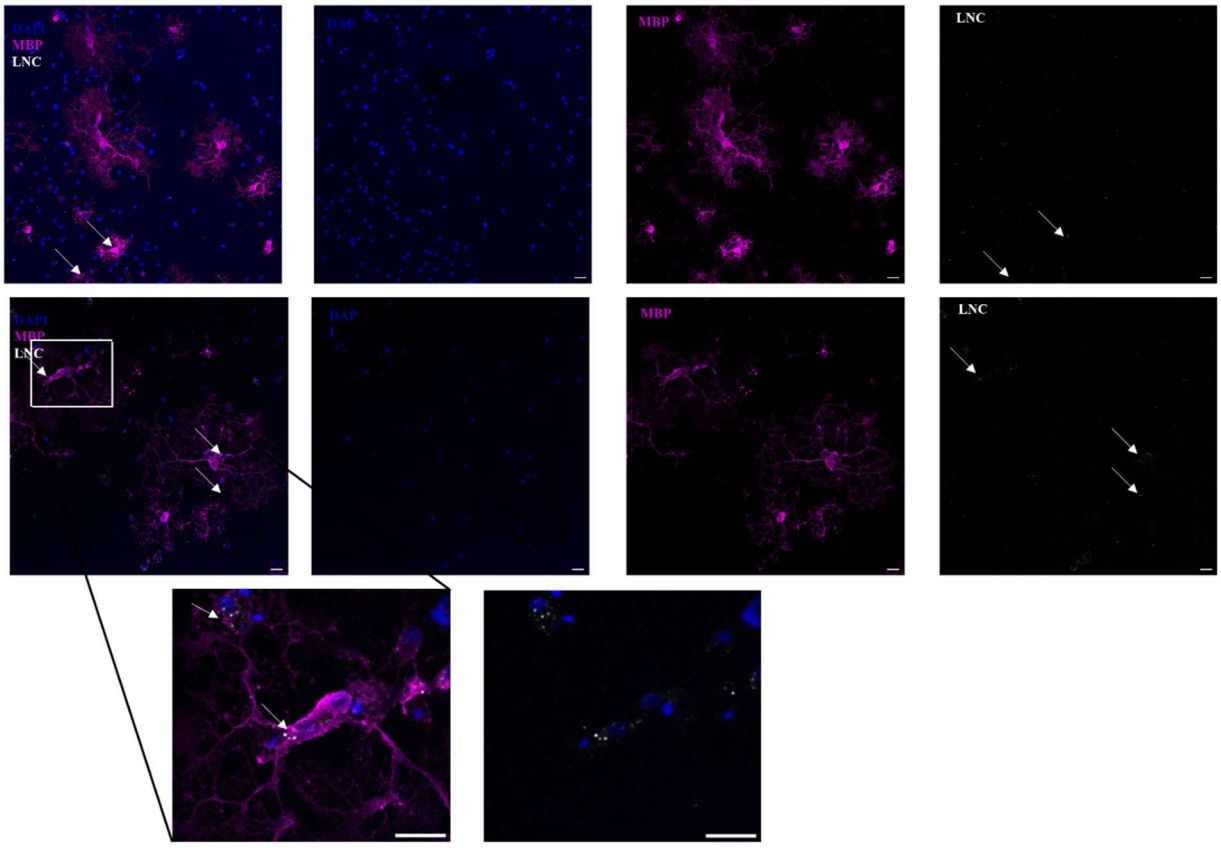

Figure 7 – Confocal fluorescence microscopy of the internalization TfRp-SPION-LNC DiD by OPCs after an incubation period of 4 hours. Day 2 has OPCs with a lower degree of differentiation, already with LNC inside, while day 5 has late stage OPCs, with LNC placed close to the cell nucleus, indicating the LNC involvement from an early stage. The arrows indicate the presence of the TfRp-SPION-LNC DiD. Magenta: MBP, Blue: Hoescht. Scale bar = 20 µm

3.2.3 – Permeability of the lipid nanocapsules across a blood-brain barrier monolayer

To understand if LNC nanoparticles were able to permeate the physiological barrier that is the BBB, a well-established in vitro model of the BBB was reproduced [16]. This model based on a hCMEC/D3 cell line has several advantages over other BBB in vitro models, including the i) maintenance of BBB-specific properties over passages; ii) simple to maintain and low cost to reproduce; and iii) the capacity of forming functional barriers with key tight junction proteins [49].

In order to verify the potential for receptor-mediated transcytosis of TfRp-SPION-LNC RA, the concentrations with the best results were used on the uptake and the viability assays (0.1 and 1 $\mu g.mL^{-1}$ of RA), and the formulation was evaluated at 60, 120, 180 and 240 minutes. The permeability coefficients were similar among conditions (Figure 8), but the external functionalized with TfRp improved the permeability of the LNC (approximately 0% for the free drug and 3% for the functionalized LNC) after the 4 hours. Throughout the assay, it was observable that all formulations experienced a linear growth in the quantities that permeated the monolayer, but the functionalized LNC demonstrated the highest values. A significant finding was the degradation observable by RA under these conditions (Table S3), wherein the encapsulation could result in some protection to ensure the cargo reaches the intracellular compartments to perform its biological activity.

These results are in line with reported applications of LNC for brain delivery, where the permeability coefficients were improved by external functionalization [41]. The improved delivery rates were also verified in other works when exploiting the transferrin pathway [16, 50, 51]. Having this in mind, this work suggests that this versatile platform is able improve the delivery rates of lipophilic drugs to the CNS, unlocking novel therapies that were once not feasible for administration due to poor bioavailability. The TEER remained intact throughout the assay (Figure S4).

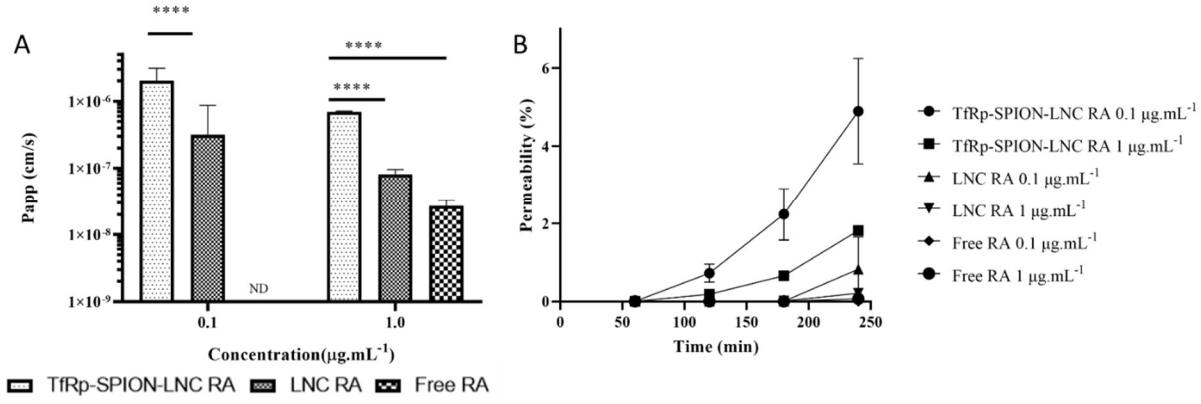

Figure 8 – Permeability of functionalized LNC compared to non-functionalized LNC and the free drug. (A) is the Papp at 4 hours through a monolayer of hCMEC/D3 cells, (B) is the

permeability kinetics through 4 hours. Results from N=3 triplicates; Mean +/- SD, ****p<0.0001; ND = not detected.

3.3- Anti-inflammatory and oligodendrocyte-progenitor cells differentiation potential of lipid nanocapsules

The two main events on demyelinating disease such as MS are the loss of the myelin sheath of neurons produced by oligodendrocytes and the aggressive inflammatory milieu. Microglia particularly undergoes significant activation [52], contributing to a widespread inflammatory environment in lesion sites that together with astrocytic activation eventually leads to the formation of a glial scar [53].

To understand if TfRp-SPION-LNC RA are able to hold an anti-inflammatory activity when in contact with microglia, and to decrease their activation state, BV-2 microglia cells were firstly activated by the presence of LPS for 1 h. When microglia undergo activation in response to an external stimulus, it undergoes morphological alterations that are easily observable, particularly adopting an ameboid, circular conformation rather than an elongated, branched phenotype [54]. Also, the conformation alterations are coupled with phenotypic changes, resulting in the production of pro-inflammatory cytokines. With the presence of RA after the exposure to this stimulus, the microglia can revert back to its normal conformation, drastically reducing the production of inflammatory markers and the ability to establish an inflammatory, aggressive environment [55]. To quantitatively verify if there is an actual suppression of pro-inflammatory stimuli and a shift towards an anti-inflammatory state, IL-6, TNF-α and NO were chosen as pro-inflammatory markers, and IL-10 was chosen as an anti-inflammatory indicator. The expression of these markers was quantified by ELISA (IL-6, TNF-α and IL-10) and the Greiss reaction (NO) as represented in Figure 9. To study a potential dose-response effect, three different concentrations (0.01, 0.1 and 1 $\mu g.mL^{-1}$) were selected based upon the metabolic activity previously demonstrated in order to try to establish an active range of concentrations. Blank-LNC, free RA (equivalent to the highest concentration of LNC RA, 1 $\mu g.mL^{-1}$) were used as controls to compare the anti-inflammatory action with TfRp-SPION-LNC RA on LPS stimulated cells. Non-stimulated cells were used as a negative control for the presence of anti-inflammatory biomarkers.

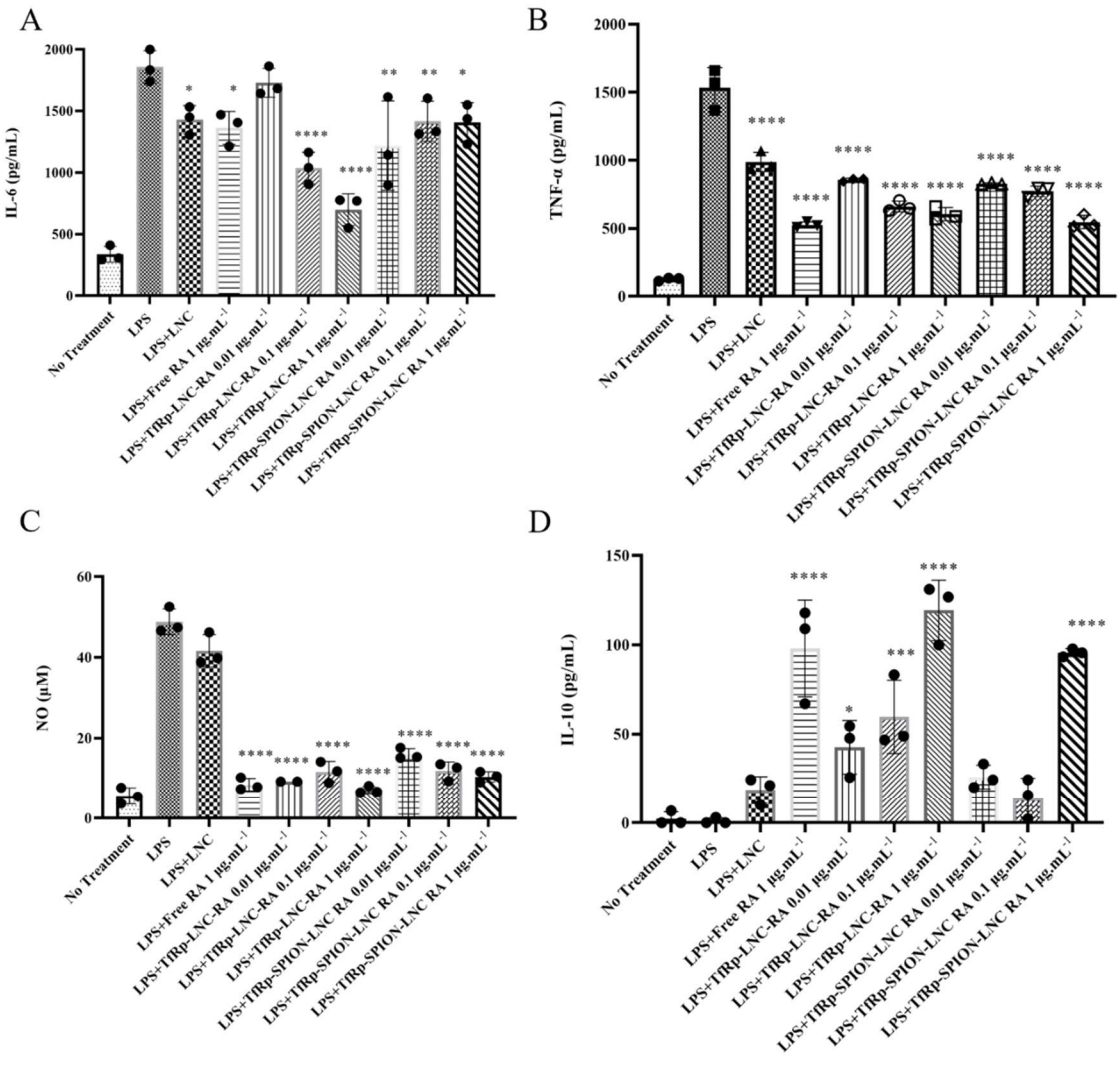

Figure 9–- Analysis of inflammatory biomarkers to verify if TfRp-SPION-LNC RA are able to establish its anti-inflammatory activity and to suppress microglia activation. A, B and C are pro-inflammatory mediators, whilst D is an anti-inflammatory cytokine, showing that RA can play both sides of the inflammatory cascade. The comparisons were always performed versus the LPS only well. Results from n=3 independent experiments (two duplicates per condition); Mean +/- SD, *p<0.05; **p<0.01; ***p<0.005; ****p<0.0001.

From the results of ELISAs and the Griess assay it is observable that RA and LNC RA have a potent anti-inflammatory action, acting on the production of NO and the cytokines IL-6, IL-10 and TNF-α. The results are in accordance with previously reported studies on the role of RA [56, 57]. Here, LNC was able to preserve the activity of RA, matching up, or outperforming the free RA. All tested concentrations of RA within the LNC exhibited anti-inflammatory activity, with the highest (1 $\mu g.mL^{-1}$) exhibiting the most marked effect. This was particularly notorious on the production of the anti-inflammatory cytokine, IL-10. The concentrations of 0.01 and 0.1 $\mu g.mL^{-1}$ produced 5 to 6-fold less IL-10 than 1 $\mu g.mL^{-1}$ which highlighted the concentration dependent effect of RA. Interestingly, for IL-6, free RA did not have the same reduction compared to the LNC RA. This could be explained by the premature degradation of RA before it interacts with the cells, reinforcing the need for a vector to deliver this molecule. The presence of SPIONs within the LNC also did not impair the activity of RA or affected the cells in any way. The suppression of these biomarkers is in line with the mechanistic action of RA, acting through its target, the retinoid-x-receptor (RXR), and has been successfully demonstrated before as being an effective inhibitor of microglia activation [43, 58]. This action occurs not only due to the effect of RA, but also its isomers such as 9-cis-RA [58]. Our results are in accordance with these previously reported studies in the literature, suggesting that TfRp-SPION-LNC RA is a viable candidate to deliver RA to the CNS.

After demonstrating that LNC RA is able to significantly reduce the inflammatory environment, it was important to verify if it could significantly promote the differentiation of OPCs into mature, myelin producing oligodendrocytes. The differentiation stage of an oligodendrocyte represents the degree of maturity it has reached, and the higher the stage, the higher ability to produce higher quantities of myelin [59]. Thus, to verify if LNC RA is able to improve the differentiation of OPCs we observed the expression of MBP, the main indicator for the presence of myelin, and oligodendrocyte branching based on the occupied area by the tubulin protuberances.

To start, the differentiation profile of oligodendrocytes was evaluated. The impact of nanoparticles on the number of MBP cells and area occupied by each oligodendrocyte was assessed recuring to automated image analysis using open-source software. These outputs positively correlate with the ability of the cells differentiating towards a myelinating/mature state. As expected, our results show that the number of MBP positive cells increased from day 2 to

day 5 of differentiation suggesting that cells are able of continuing with their intrinsic differentiation profile (Figure 10A). Additionally, the presence of the particles did not affect the number of cells capable of producing the myelin marker MBP (Figure 10A). Nonetheless, tendentially increased cell areas for the formulations containing RA were observed for both time-points, suggesting that the presence of RA tends to originate larger oligodendrocytes (Figure10B, i.-viii.).

The higher capability of RA formulations to increase oligodendrocytes' area is directly linked to the mechanism of action of RA through direct stimulation of its target receptors, notably the RXR and retinoid-A-receptor (RAR). Both receptors share similar signal transduction pathways but differ in the ligands that are able to activate them. Stimulation of these receptors has been linked particularly to improved differentiation of OPCs both in vitro and in vivo [23, 60]. Particularly, direct stimulation of RXR-γ in aged rats where demyelination was induced resulted in an increase in the number of myelinated neurons, and a slight increase in MBP+ cells [61]. Another study demonstrated that mice that were knockout for the retinoid receptors experienced a delay in the recruitment of OPCs and their subsequent differentiation. Also, the mice demonstrated thinner myelin then the ones that were treated with bexarotene [62]. Although our results were not able to significantly demonstrate an increase of the differentiation of OPCs, there is a significant trend implying that the presence of RA tends to improve the overall performance of OPC differentiation.

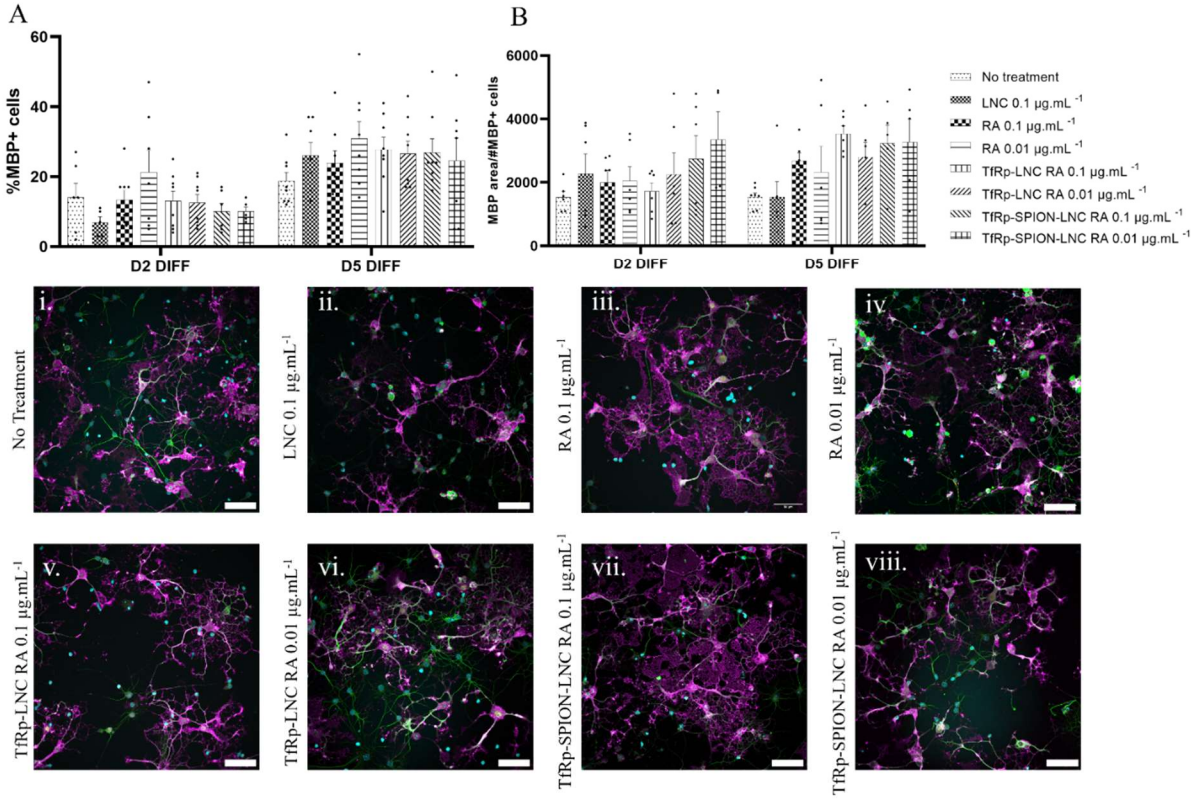

Figure 10–- MBP expression and quantification after high-throughput image screening. (A) represents the calculated expression of MBP in cells identified to be Olig2 positive (oligodendrocyte line). (B) represents the calculated ratio of the area of occupied by MBP divided by the MBP+/Olig2+ positive cells. Results are from n=4 independent experiments (two replicates per condition). Data is shown as Mean +/- SD after outlier evaluation. (i-viii) Representative confocal microscopy images of each condition at day 5 of differentiation. Magenta: MBP, green: actin, yellow: Olig2, cyan: Hoechst. Scale bar = 50 µm.

To evaluate in depth the oligodendrocyte branching ability, oligodendrocytes were categorized in four differentiation stages according to a previously established protocol (“collar occupancy”) [33]. Stage I corresponds to mono- or bipolar OPCs; in stage II OPCs are primary branched with three or four processes; in stage III are secondary branched with 4-5 main processes; and stage IV corresponds to fully mature, extensively branched oligodendrocytes with membranous

processes (Figure 11 i-iv.). By estimating the area of the tubulin staining (which corresponds to protrusions of the oligodendrocytes) occupying a defined area around the nucleus (“collar occupancy”) a direct relation can be established with the differentiation stage of the oligodendrocytes [33]. Here, the presence of RA, whether encapsulated within the LNC or in the free form, led to a distinct increase of late-stage oligodendrocytes at day 5 of differentiation, suggesting the ability of RA to promote oligodendrocyte differentiation. This could point towards the potential for the LNC to promote the internalization of RA to interact with its nuclear receptors before it can be degraded. This data directly correlates with the results from the MBP expression and is in line with previously reported results regarding the role of RA on the differentiation of OPCs [63]. It was shown that synthetic agonists of the RA receptors (RARβ and RARα) improved myelination and differentiation of oligodendrocytes in NG2+ cells [60]. However, it should be noted that this study also highlighted prolonged treatment with RA may begin to induce the upregulation of specific genes that may hinder OPC differentiation [64]. Thus, when designing a treatment focused upon RA for the differentiation of OPC the course of the treatment be warranted to avoid a negative effect from long-term treatment.

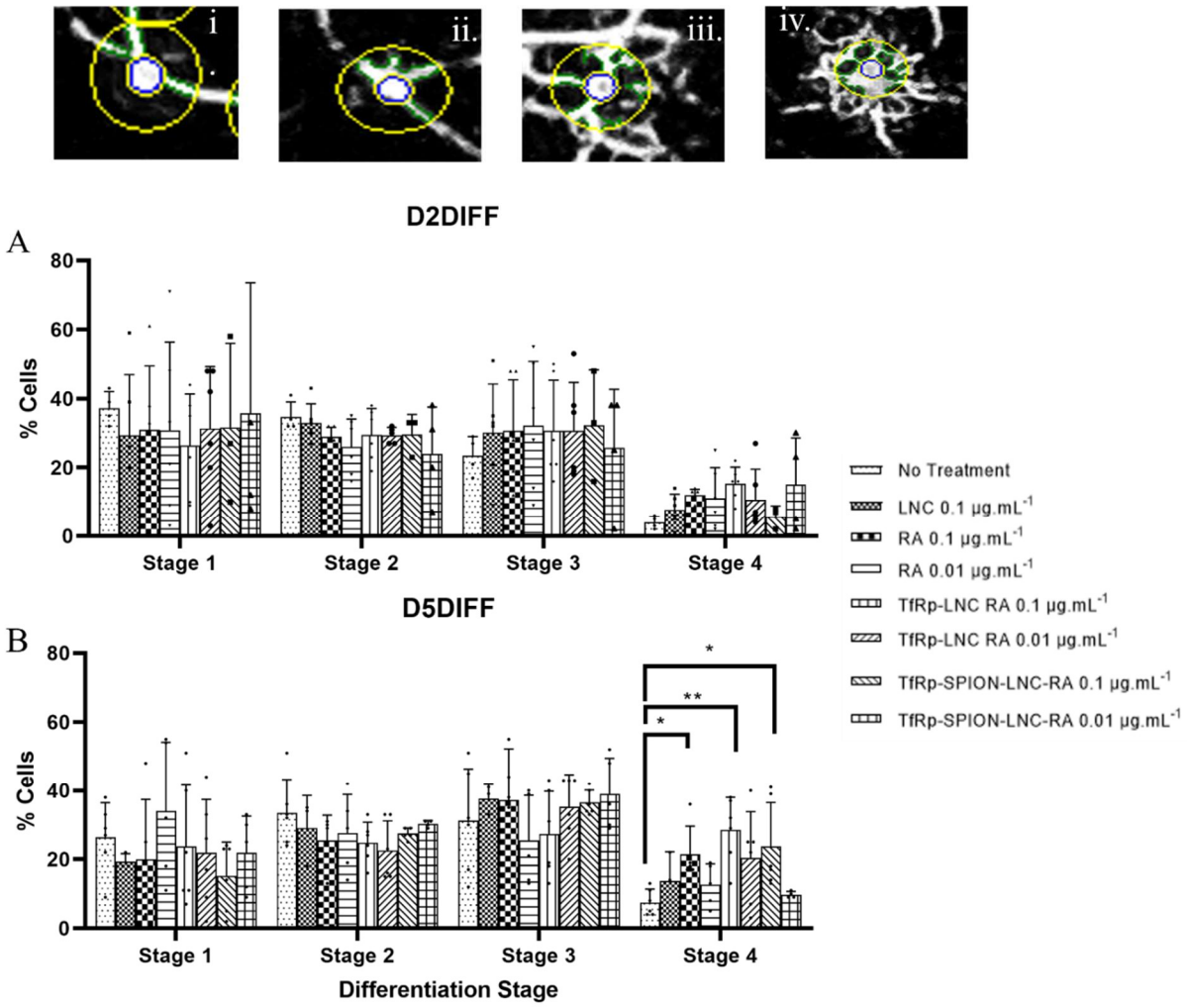

Figure 11–- Collar Occupancy analysis of the differentiation stages of OPCs. (A) are the results from the 2nd day of differentiation, while (B) is the results from the 5th day of differentiation. The cells are classified on their stages based on the area that the protrusions from the nuclei occupies relating to the area of the nuclei, as demonstrated on images (C-F). (C) is stage I; (D) is stage II;€) is stage III; and (F) is stage IV. Results are from n=3 independent experiments (two duplicates per condition). *p<0.05; **p<0.01.

The use of LNC has already been established and demonstrated as effective carriers to interact with OPCs, both in vitro and in vivo [2, 27]. Particularly, in vivo LNC demonstrated the ability to restore the percentage of mature oligodendrocytes to pre-lesion levels in a mice model of focal demyelination through a stereotaxic injection 7 days post treatment [2]. Despite this, not many nanotechnology-based approaches to target OPCs have been used in the literature. A work compared the effectiveness of liposomes to polymeric particles to extracellular vesicles, and it demonstrated that despite lower uptake values, extracellular vesicles showed the best ability to induce OPCs differentiation. As LNC more closely resemble a carrier vesicle, but also benefit from some of the properties of liposomes and polymeric nanoparticles, such as better cargo loading and stability, as well as the use of biocompatible excipients, they could prove to be an efficient carrier to target OPCs [65]. Despite this, there is still a critical necessity to improve the studies and the overall study design in approaches to target OPCs to attempt to develop an optimal system to tackle demyelinating pathologies.

4. Conclusions

In this work, a BBB-targeted LNC-based nanosystem was developed and its impact on the metabolic activity and interaction of target cells evaluated, as well as on disease associated relevant parameters, aimed at tackling MS. Particularly, monodisperse LNCs were developed, and surface functionalized with the addition of TfRp. The aim of this strategy to promote active brain targeting resulted in a 3-fold increase in the uptake by BBB endothelial cells, which can be further potentiated by the coupling with the encapsulation of SPIONs for the potential magnetic targeting in in vivo models. This is a candidate strategy that can be translated to other types of LNCs that could significantly improve brain delivery of therapeutics. The LNCs demonstrated a dose-dependent cytotoxicity when in contact with microglia and endothelial cells with a safe concentration range of around 1μg.mL^{-1} of encapsulated RA. We successfully demonstrated that encapsulation within the functionalized LNC resulted in significant improvement of the permeability profile of RA through the BBB and demonstrated that the LNC are able to be internalized by endothelial cells, microglia and OPCs in order to exert its action. Following that, the LNC demonstrated the ability to preserve the bioactive role of RA when in contact with microglia, resulting in suppression of three different pro-inflammatory cytokines by

5-6-fold compared to no treatment at all and a 100-fold improvement on the production of the anti-inflammatory cytokine IL-10 was also demonstrated. Regarding the OPCs, the LNC were able to significantly improve by 2-3-fold the presence of late stage, mature oligodendrocytes that are more capable of producing higher quantities of myelin, and demonstrated improved performance when compared to the free RA. There was also a noticeable trend on the amount of MBP+ cells and the area occupied by them when RA was present in the formulation. The particular significance of this results is because RA lacks the ability to reach the brain in significant concentrations due to premature degradation and a necessity of a specific carrier protein to circulate in the bloodstream. Thus, the TfRp-functionalized LNCs coupled with the SPIONs nonetheless act as a versatile carrier that could encapsulate other types of lipidic drugs that could be used to achieve higher concentrations at the CNS, in the scope of neurodegenerative diseases. Further work should be carried out now to validate the nanosystem in vivo, to confirm the improved targetability towards the brain by the co-encapsulation of SPIONs, and to establish a proof-of-concept in a demyelinating disorder model.

5. Supplementary Information

Table S1 – Elemental analysis of TfRp-SPION-LNC RA and unfunctionalized LNC to check for presence of TfRp-Cys after dialysis. Results are from n=1 representative assay.

Formulation	Sulphur %
LNC	0%
SPION-LNC RA	0%
TfRp-SPION-LNC RA	0.04%

Table S2 – Association efficiency of TfRp-SPION-LNC RA stored at 4ºC over time. Results are from *n=3* experiments (two duplicates per condition), presented as Mean +/- SD.

Timepoint	Size (nm)	PdI	Zeta-potential (mV)	RA Association efficiency (%)
0 months	71 ± 3	0.125 ± 0.021	-3.81 ± 1.20	-
3 months	74 ± 1	0.094 ± 0.030	-4.56 ± 0.39	88.8 ± 2.0
6 months	72 ± 1	0.096 ± 0.015	-4.57 ± 0.44	89.7 ± 1.9
9 months	76 ± 2	0.131 ± 0.071	-4.13 ± 0.69	80.1 ± 5.3

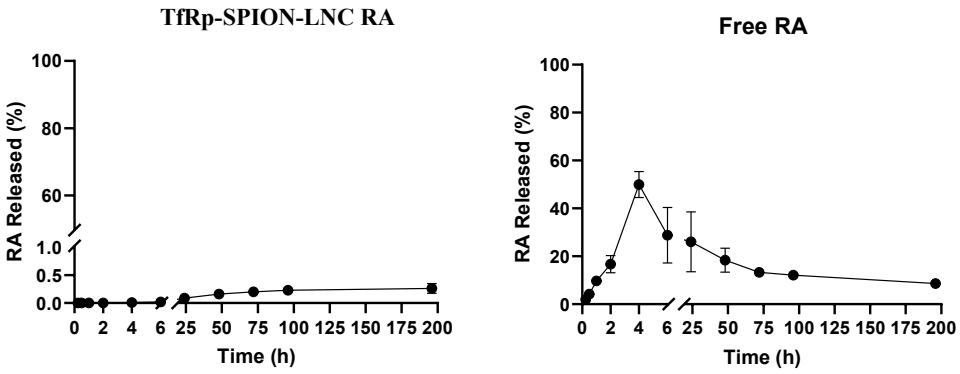

Figure S1 – Release profile of RA from LNC compared to free RA in simulated biological media (PBS pH 7.4 with 1% Tween-80). We found that at 4 hours it was visible that RA was degrading significantly, as is observable in the graph. The TfRp-SPION-LNC RA exhibited practically no release after 8 days, suggesting that the LNC only release their payload when in contact with cells (either by direct LNC-cell membrane fusion or by the conditions experienced within endosomes. Results are from *n*=3 experiments (with triplicates per condition), presented as Mean +/- SD.

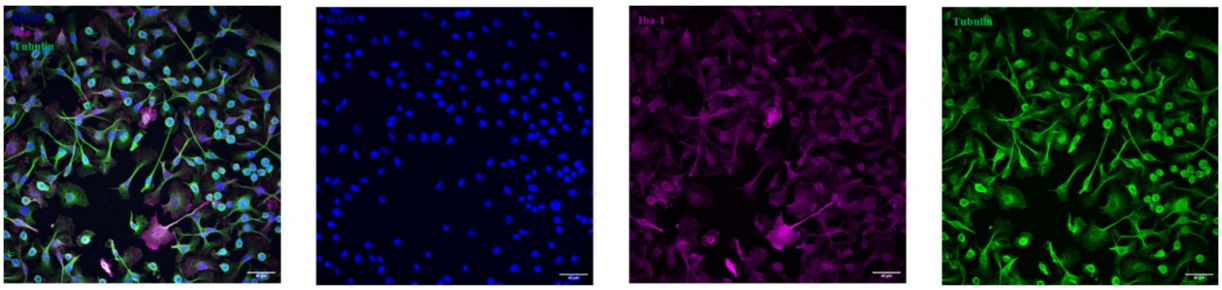

Figure S2 – Confocal fluorescence microscopy of the internalization of the BV-2 microglia after an incubation period of 4 hours. This was performed as the untreated control version of the uptake assay to demonstrate the LNC do not activate the microglia by themselves, preserving their rested state. Magenta: Iba-1, Green: Tubulin, Blue: Hoechst. Scale bar = 40 µm.

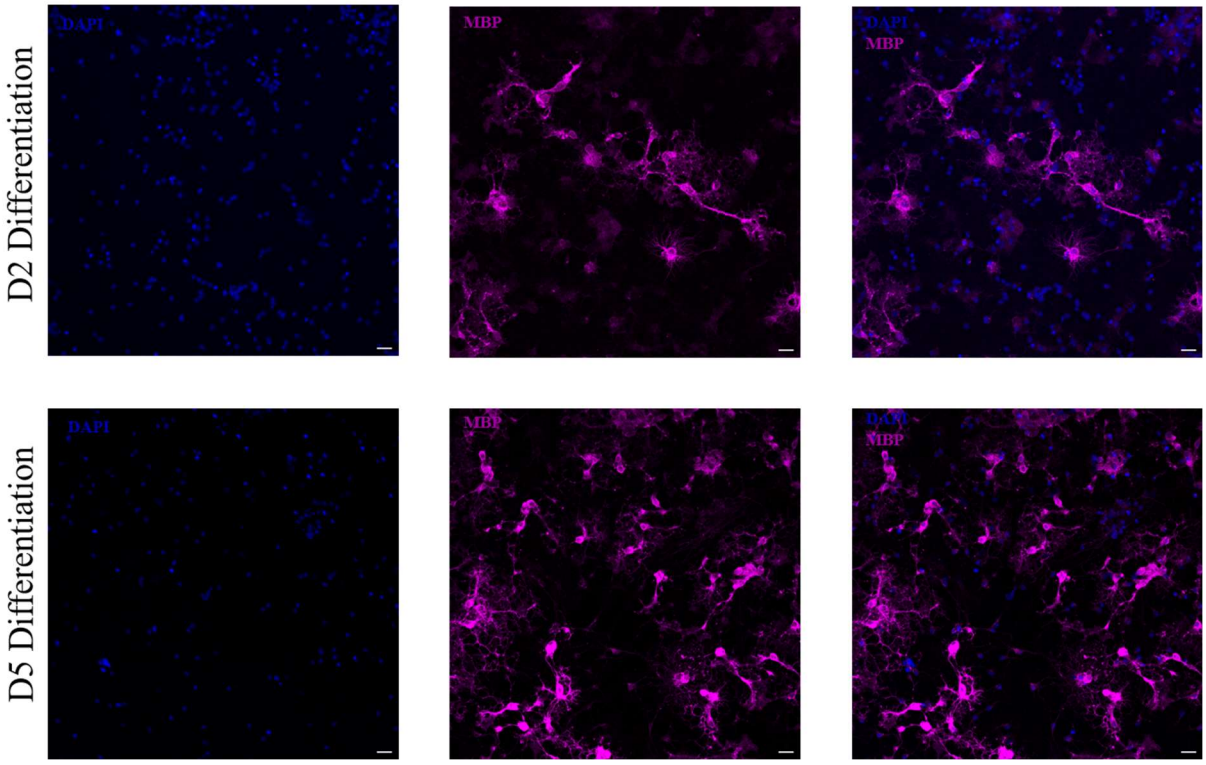

Figure S3 – Confocal fluorescence microscopy of the internalization of the OPCs after an incubation period of 4 hours. This was performed as the untreated control version of the uptake assay to demonstrate the LNC do not impact the OPCs. Magenta: MBP, Blue: Hoechst. Scale bar = 20 µm.

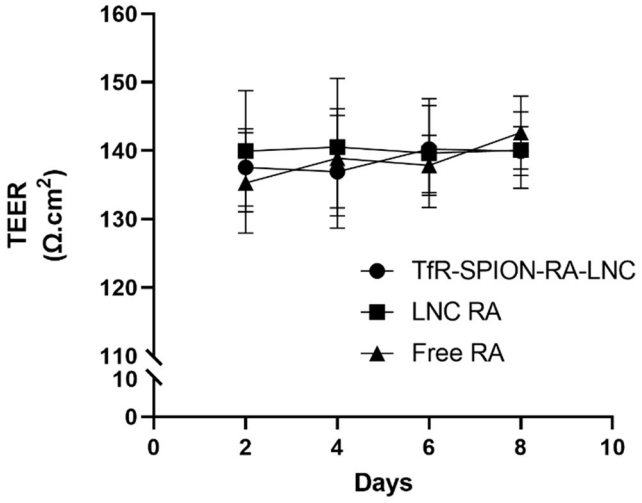

Figure S4 – Trans-endothelial electrical resistance (TEER) measurements of hCMEC/D3 cell monolayer from seeding to assay day. Results are from N=3 triplicates.

Table S3 – Degradation profile of RA when at 37ºC for 4 hours, measure by HPLC. Results from N=3 triplicates, presented in mean %.

Timepoint (hours)	**% of RA**
T0 in acetonitrile	100%
T0 in HBSS	100%
T4 in acetonitrile	61.6%
T4 in HBSS	55.4%

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CHAPTER– 4 - RETINOIC ACID LIPID NANOCAPSULES IMPACT IN AN IN VIVO MICE MODEL OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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The impact of retinoic acid lipid nanocapsules in an *in vivo* mice model of experimental autoimmune encephalomyelitis

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Abstract

Multiple sclerosis is a debilitating, chronic disease that currently affects millions of people worldwide. There is yet a therapeutic approach that can successfully halt the progression of the disease and restore the destroyed myelin at the central nervous system. Here, we aim to demonstrate that functionalized lipid nanocapsules (LNC) loaded with retinoic acid (RA) is a capable system to transiently suppress and limit the progression of multiple sclerosis. To accomplish this, two different routes of administration (intravenous and intranasal) were compared to select the one with the highest brain accumulation, where intravenous showed the highest accumulation at the brain. Results showed that transferrin functionalization seemed to hinder the lipid nanocapsule accumulation in the brain and induce unwarranted toxicity in mice. In an experimental autoimmune encephalomyelitis (EAE) model, intravenous LNC showed that there were no significant differences regarding the clinical score of mice treated with retinoic acid LNC and no treatment whatsoever. There was, however, a 2.5-fold reduction in the fluoromyelin unstained area with the retinoic acid nanocapsules, suggesting that there is a potential for the particles to either restore damaged myelin or prevent focal demyelination in multiple sclerosis.

Keywords: Experimental Autoimmune Encephalomyelitis; Multiple Sclerosis; Neurodegeneration; Myelination.

1- **Introduction**

Amongst central nervous system (CNS) diseases, neurodegeneration, particularly when characterized by a progressive loss of myelin, usually results in significant comorbidities for the patients [1]. Amongst demyelination disorders, they can hold similar cellular events, such cycles of progressive worsening of the lesion sites in the CNS, with asymptomatic periods. Despite this, even during remission periods, neurodegeneration can still progress further. During an active flare-up of the inflammation, the regular function cellular constituents of the brain (astrocytes, neurons, oligodendrocytes) are significantly impaired, resulting in a loss of function of these, or an improper activation towards harmful phenotypes (microglia) [2]. In multiple sclerosis (MS), oligodendrocytes are particularly affected and undergo apoptosis during relapses [3]. Damage to these cells results in poorly insulated neurons, significantly dampening the ability of the signals to process through the axons, resulting in significant adverse effects to the quality of life of afflicted patients [4].

When considering MS, there seems to be a number of factors that can contribute towards the appearance of the disease, thus earning itself as the designation of a multifactorial disease, where a significant immune reaction occurs against certain constituents of the myelin sheath of neurons, leading to the appearance and maintenance of a persistent inflammatory state [5]. There are different hallmarks on which MS can arise in patients, ranging from genetic, environmental, or possibly infectious due to molecular mimicry [6]. When considering the early stages of the disease, it has been shown that the body has the ability to mobilize cells, that are termed oligodendrocyte progenitor cells (OPCs) that can restore the damage myelin and the destroyed oligodendrocytes, effectively contributing to a suppression of the damage caused by the disease [7]. However, within each lesion cycle the microglia and the astrocytes acquire a more reactive phenotype and form a scar around the zone, named a glial scar . [8]. The glial scar can be seen as the brains attempt at a short-term solution, but in the end, it results in long-term problems, as the scar will prevent the return of normal function of that lesion location [9].

Currently, therapeutic approaches focus on the management of the symptoms and the flare-ups of each episode [10] merely controlling the inflammatory environment, with hopes of

reducing the rate of demyelination to levels that can be replenished by the regular OPC mobilization [11]. However, the efficiency of OPC to remyelinate is affected in MS, contributing to lower rates of endogenous repair, making this process insufficient to address CNS damages MS [12]. As there is no approved treatment yet for the stimulation of remyelination and even less to cure MS, this disease is an unmet medical need. Thus, there is a need for therapies that increase the OPC ability to remyelinate the unhealthy CNS [13]. However, most remyelinating therapies that are developed in a pre-clinical setting fail to reach the clinic due to toxicity, lack of correlating results from *in vitro* to *in vivo*, poor biodistribution or a lack of effectiveness in randomized trials [14].

Retinoic acid (RA) is a therapeutic molecule known for its anti-inflammatory action in the CNS [15, 16], but also for the stimulation of the differentiation of precursor cells and stem cells [17-19]. As RA is capable to act on both fronts of MS, it is an ideal candidate that could not only dampen the CNS inflammatory environment but improve endogenous remyelination. However, RA cannot cross accumulate in the CNS at therapeutic concentrations [20]. Thus, using nanotechnology to solubilize and transport RA through the BBB, an emerging opportunity appears to reach adequate RA concentrations in the CNS [21]. We have previously demonstrated that LNC loaded with RA [22] can cross the BBB and exert RA dual-effect action, suppressing the inflammation at the CNS and promoting the differentiation of more mature oligodendrocytes [23].

Herein, the goal of this work was to evaluate the therapeutic impact of LNC RA in a mouse relevant pre-clinical model of MS, the experimental autoimmune encephalomyelitis (EAE). This work aimed 1) to carry on the *in vitro* validation of the nanosystem, 2) to assess the LNC biodistribution and accumulation in the CNS of healthy mice comparing two different routes of administration, 3) to identify potential off-target effects to choose a route of administration for the nanosystem and lastly 4) the evaluation of LNC RA impact on the clinical score and myelination of EAE mice.

2- **Materials and methods**

2.1- Materials

Transferrin-receptor binding peptide (Thr-His-Arg-Pro-Pro-Met-Trp-Ser-Pro-Val-Trp-Pro-Cys) was purchased from GenScript (Piscataway, NJ, USA); crosslinker (NH_2-PEG-Mal, PEG of 5 kDa) from Biochempeg (Watertown, MA, USA) Labrafac® was kindly provided by Gattefosse SA (Saint-Priest, France); Phospholipon® 80 H Lipoïd® was purchased from Lipoid Gmbh (Ludwigshafen, Germany); Kolliphor® HS 15 was gifted from BASF (Ludwigshafen, Germany); DiD solid; DiIC18(5) solid and phalloidin-alexa fluor 488 was bought from Alfagene (Lisboa, Portugal)'1,1'-dioctadecyl-3'3,'',3'-tetramethylindotricarbocyanine iodide (DiR) was purchased from Thermo Fisher Scientific (Bleiswijk, The Netherlands); iron oxide(II,III), magnetic nanoparticles were acquired from Laborspirit, lda (Lisboa, Portugal); hematoxilin, eosion, retinoic acid, millex-HV Syringe Filter Unit, 0.45 µm, sodium chloride, sodium hydroxide, glycerin, paraformaldehyde, resazurin, sodium nitrite and sodium azide were purchased from Sigma (St. Louis, MO, USA); DMSO, O.C.T., isopropanol compound was purchased from VWR (West Chester, PA, USA); Reverse Transcription System and Go Taq qPCR master mix were purchased from Promega® Benelux BV (Leiden, The Netherlands); High glucose with UltraGlutamine™ Dulb'cco's Modified Eagle medium (DMEM) from Lonza (Basel, Switzerland); for high-pressure liquid chromatography (HPLC) analysis, trifluoroacetic acid (TFA) purchased from Acros Organics (USA) and Acetonitrile HPLC Gradient Grade purchased from Fisher Scientific (UK) were used; Triton X-100 from Spi-Chem (West Chester, PA, USA); fetal bovine serum (FBS), penicillin-streptomycin; ethanol from Valente e Ribeiro, Lda (Lisbon, Portugal); Hank's Balanced Salt Solution (HBSS) without calcium or magnesium supplemented with trypsin (0.0025% w/v) and 0.001mg.mL^{-1} DNAse I was purchased from Applichem GmbH (Darmstadt, Germany); Por Float-a-lyzer G2 10 kDa dialysis membrane and Rotilabo® syringe sterile 0.22 µm filters were purchased from Carl Roth GmbH (Germany); Ultrapure water was prepared in-house.

2.2 – Lipid nanocapsules

LNC were prepared following the protocol as described previously [23]. In brief, Kolliphor® HS 15 (0.450 g), Phospholipon® 80 H (0.045 g), NaCl (0.060 g), Labrafac® (0.600 g) and water (1.975g) were mixed with gentle magnetic stirring at 40 ºC for 10 minutes. Afterwards, the

solution was slowly heated until 90ºC and cooled to 60 ºC a total of three times. During the last cycle, a rapid shock with cold water (10 g, at 4ºC) was added at 75-77 ºC under high-speed stirring. For RA loaded LNC, 250 µL of previously dissolved RA in DMSO (10 $mg.mL^{-1}$) was added at 79-81ºC at the last heating/cooling cycle. For dual-encapsulating DiR-LNC RA, DiR was dissolved to a concentration of $1mg.mL^{-1}$ in absolute ethanol and evaporated under an inert atmosphere before incorporating in the LNC formulation. For blank LNC, 250 µL of water was added instead. Afterwards, the LNC were centrifuged at 3200 x g to remove all the unreacted lipids and then filtered through a 0.45 µm syringe filter. All formulations were then stored at 4ºC until further use. When applicable, the formulations were then functionalized following the protocol described in [23], based on the transacylation process of the surface of the LNC and the post-manufacturing inclusion of a cysteine carrying peptide (TfRp-Cys).

2.3 – Impact of LNC on blood clotting

The impact of the LNC on coagulation was studied by a clotting assay. Normal human plasma was obtained from healthy volunteers (Centro Hospitalar de São João, EPE, Porto, Portugal). Aliquots were stored at -20°C until use. Functionalized and unfunctionalized LNC RA, free RA and vehicle (0.9% saline) were added to the wells of a 96-well plate. Glass coverslips with saline were included as a positive control. A 1M stock solution of calcium chloride was added to the thawed plasma to a final concentration of 20 mM to initiate clotting. Immediately, recalcified plasma was vortexed and added to the 96-well plate. The plates were then placed in an incubator at 37 ºC with mild agitation (150 rpm). They were then measured every minute with a multiplate reader (Biotek® Synergy Mx) by a kinetic assay using absorbance at 405 nm. The clot times were identified when the absorbance reached a maximal level, corresponding to the time it took for the plasma to jellify.

2.4 – Impact of LNC on Human nasal epithelial cell (RPMI 2650)

2.4.1 – Cell culture

Human nasal epithelial cells (RPMI 2650) were grown in tissue cultured flasks, in Dulbecco's modified eagle medium (DMEM) supplemented with FBS (5 %, v/v), penicillin-streptomycin (1

%, v/v). Cells were sub-cultured every 3–4 days using trypsin–EDTA. The culture medium was replaced every other day. Cells between passages 22-28 were used.

2.4.2 – Metabolic activity

Cells were seeded in a 96-well plate at a density of 6.25×10^4 viable cells/cm^2. The cells were then incubated for 24 hours and washed twice with phosphate buffered saline (PBS). Afterwards, the cells were incubated with different concentrations of blank-LNC, free drug and TfRp-LNC RA, for 15 minutes and 1 hour. The cells were incubated with 10% (v/v) solution of resazurin diluted in DMEM as per supplier 'instructions for 3 hours. Fluorescence was measured at an excitation of 530 nm and emission of 590 nm using a fluorometer Synergy Mx (BioTeK Instruments, GenX software).

2.4.3 – LNC transport across a nasal epithelium *in vitro* model

A nasal epithelium *in vitro* model was developed to evaluate the permeability of the LNC. Briefly, 2.5 x 10^4 viable cells/cm^2 of RPMI 2650 cells (500 µL of medium) were seeded in 12-well plates in transwell® cell culture inserts with a 1 µm pore size. The system was maintained for 21 days during which medium was changed every 2 days. The RPMI 2650 cell monolayer became confluent at the day 21 after seeding, which was the day appropriate to perform the permeability study. The integrity of the cell monolayer was checked every 4 days by monitoring the trans endothelial electric resistance (TEER) using an endothelial Volt–Ohm meter (Millicell® ER S-2; Merck Millipore, Billerica, MA, USA). The resistance value of an empty filter with only media was subtracted from each measurement.

The permeability experiment was performed in the apical-to-basolateral direction. After removing the cell culture medium, the basolateral compartment was filled with 1.5 mL of HBSS, ensuring sink conditions. RA, at 0.1 and 1 µg.mL^{-1}, in solution or loaded in LNC was added at the apical side. Then, the assay was conducted at 37 ºC using an orbital shaker incubator (100 rpm). At different time points (15, 30, 45 and 60 min), 200 µL were taken from the basolateral side and were replaced by fresh pre-heated HBSS. At the end of the experiment, the cell monolayer was lysed using 1% Tween-80 to quantify intracellular RA. The integrity of the cell monolayer was checked during the permeability experiment by monitoring the TEER (values >

30 Ωcm2 were considered for the experiment). The apparent permeability coefficient, Papp ($cm.s^{-1}$) was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

where dQ (µg) is the amount of drug that permeated from the apical to the basolateral compartment during the incubation time t (s), A is the surface area of the transwell® insert (cm^2) and C_0 the initial concentration of the drug on the apical side ($\mu g.mL^{-1}$). The samples were analyzed by the method described previously [23]. For the quantification, 20 µL of each sample was separated on an HPLC Vanquish (Thermo Fischer Scientific, Bremen, Germany).

2.5 – In vivo biodistribution and toxicity of LNC RA

C57/BL6 mic– (8 - 9 weeks) (Janvier) were housed in filter-top cages under artificial light (12 h dark/12 h light) and controlled conditions of temperature (20–22 °C) and had free access to food and drinking water. In vivo experiments were performed following the Belgian national regulation guidelines in accordance with EU Directive 2010/63/EU and were approved by the ethical committee for animal care of the Université Catholique de Louvain medicine faculty (2023/UCL/MD/024) unless stated otherwise.

Fluorescent LNC RA-DiR (0.3mM of equivalent RA) were administered intranasally or intravenously (20 µL) every other day for 1 week to anesthetized mice (isoflurane), followed by a sacrifice on the last day to collect the organs and to image them *ex vivo.* The animals were weighed every day to follow the impact of the treatment on their well-being.

Mice were divided into five groups: vehicle, LNC RA-DiR intranasal, TfRp-LNC RA-DiR, LNC RA-DiR intravenous and TfRp-LNC RA-DiR (n=5, N=2) and were treated either for 1or 2 weeks. DiR fluorescence imaging (FLI) monitoring was carried out to measure brain and whole-body accumulation 1 h after each administration using an IVIS® Spectrum imaging system (Perkin Elmer, USA). Furthermore, brain, cranial base (bone part that supports the brain and holds the cribriform plate) and organs were collected 1 h post administration to verify the biodistribution of the LNC. Then, the LNC accumulation was measured by *ex vivo* DiR FLI monitoring.

Fluorescence was quantified in relative radiant efficiency units (fluorescence emission radiance per incident excitation power), standardized to the control group.

The spleen, liver and kidney were then stained with hematoxylin and eosin (H&E) to verify the structural integrity of the organs. Blood was collected from the ocular vein, and then centrifuged (3000 g, 15 min at 4ºC). The plasma was then separated and analyzed for specific enzymes that suggest organ toxicity (AST/ALT/BUN/Creatinine) using a blood chemistry analyzer, DRY-CHEM NX500, Fujifilm.

2.6 - EAE mouse model

EAE was induced in 9 weeks old C57BL/6J female mice (Janvier) (12 mice/group) using a Hooke kit (Hooke Laboratories, Inc, USA) according to the manufacturer's instructions as we reported previously [24]. Briefly, mice were injected subcutaneously (2x 100µL) with 100µg of MOG35-55 in complete Freund Adjuvant (CFA) containing 2.8 mg/ml of killed M. Bacterium tuberculosis (H37RA), at two sites at the back. Two hours after immunization and the following day, mice received pertussis toxin (PTX, 100ng/100µL) in phosphate buffer saline (PBS) intraperitoneally. Control mice received CFA emulsion without the immunizing peptide on day 0 and PTX vehicle on days 0 and 1. LNC RA (0.3mM of RA), blank-LNC (equivalent to 0.3mM of RA) or vehicle were administered intravenous every other day under isoflurane anesthesia, starting at day 10 post immunization. Mice were weighed daily and monitored for neurological symptoms associated with a progressive paralysis of the tail and hind legs. The scoring was performed by an experimenter blinded to the treatment allocation as we described previously [24]. In brief, the scoring was as follows: Score 0: No sign of the disease; score 0.5: when picked up at the base, the tip of the tail is limp; score 1: when picked up at the base, the tail is limp; score 1.5: the tail is limp and walking is slightly wobbly with weakness of one hind leg; score 2: when picked up at the base, tail is limp, legs are not spread apart or signs of head tilting when walking; score 2.5: limp tail, the two hind legs are weak or no movement in one hind leg or strong head tilt causing the mouse to occasionally fall over; score 3: limp tail with complete or almost complete paralysis of hind legs (legs are able to paddle but not to move forward of the hind hip); score 3.5: limp tail with complete paralysis of hind legs; score 4: limp tail with complete paralysis of hind leg and complete or partial paralysis of front leg. Mice were euthanized 30 days post-immunization, one hour after the last intravenous administration of

treatments or vehicle. CNS tissues and spleens were carefully collected and stored for further analysis.

2.7 - Immunohistochemistry staining and quantification.

The lumbar spinal cord of EAE mice was fixed in PFA 4% for 48 h, transferred in a 20% then a 30% sucrose solution for 24 h each, embedded in O.C.T. and stored at -80°C. Spinal cord sections were obtained using a cryostat at a thickness of 30 µm. Fluoromyelin stain was used to assess myelin loss. Briefly, cryosections were immersed in PBS and stained with Fluoromyelin and DAPI for 1 h at room temperature. Images were acquired using a 3DHistech Pannoramic P250 Flash III slide scanner (Hungary). Image analysis was carried out by a researcher, blinded to the group allocation, using CaseViewer software (3DHISTECH, Hungary). For the percentage of area occupied, a manual method was applied. Seven to eight spinal cord slices were analysed per animal, and, for each slice, the mean value was calculated to obtain a single value per animal. As for the impact in astrocytes and microglia, GFAP (GFAP-cy3, 1/300) and Iba-1 (1:1000, with the secondary Dylight 650 1/500) staining were performed respectively, and the images were analysed by a similar process.

2.8 - mRNA extraction and real-time qPCR.

Total RNA was extracted from the spinal cord of EAE mice using the Trizol reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. One µg of total RNA was used to synthesize cDNA with the GoScript Reverse Transcription System. qPCR was performed in duplicate for each sample using the GoTaq SYBR Green mix with a STEP one PLUS instrument and software (Applied Biosystems, Foster City, CA, USA) as previously described [25]. Data are normalized to a reference gene mRNA expression, the 60S ribosomal protein L19 (RPL19). We checked that our experimental conditions did not affect the expression of the reference gene for each study. Primer sequences for qPCR are listed in Table 1.

Table 1. List of primers sequences.

Gene product	Primers 5′-3′		Accession number
	Forward	**Reverse**	
TNFα	CTACTGAACTTCGGGGTGATC	TGAGTGTGAGGGTCTGGGC	NM_013693.3 NM_001278601.1
MIP-1α	AGATTCCACGCCAATTCATC	CTCAAGCCCCTGCTCTACAC	NM_011337.2
IFNγ	GTTTGAGGTCAACAACCCACAG	GCTTCCTGAGGCTGGATTC	NM_008337.4
ATF3	CGCCATCCAGAATAAACACC	CCTTCAGCTCAGCATTCACA	NM_007498.3
MOG	GCTTCTTCAGAGACCACTCTTACC	GGGATCTTCCACTTTCAACTCC	NM_010814.2

2.9 – Statistical Analysis

GraphPad Prism Version 9.0.1 was used to perform statistical analysis. One-way or two-way analysis of variance (ANOVA) were applied to analyze differences between groups. Normality was verified by the Shapiro-Wilk and Kolmogorov-Smirnov test. In case of data not following a normal distribution, a logarithmic transformation was applied and the results were analyzed by one-way ANOVA. Different groups were compared with a post hoc test (Tukey's honestly significant difference). Results are reported as mean ± SEM. Differences were considered significant at $*p<0.05$, $**p<0.01$, $***p<0.001$ or ****p<0.0001.

3. Results and discussion

3.1 LNC characterization

Carrying on the developed work in [23], LNC were developed to improve the therapeutic effectiveness of RA, through increased accumulation in the CNS. The LNC were easily reproducible although synthesized at a different location, with the overall features being presented in Table 2. The physico-chemical properties are within an acceptable range when compared with [23]. Control formulations were subjected to the same functionalization process as TfRp-LNC RA but replacing maleimide or TfRp-cys with PBS. For the biodistribution studies, RA was co-encapsulated with DiR.

As previously observed, LNC RA had a slightly smaller size than blank LNC, but their properties were otherwise maintained. When functionalizing the LNC with TfRp-cys, there was a noticeable size increase compared to controls, as well as an increase of the PdI that suggests that the functionalization process occurred as intended. The encapsulation efficiency of RA was not affected by the addition of DiR.

Table 2. LNC Physico-chemical characteristics. Results from n=3 batches, stored at 4ºC for further use.

Formulation	Size (nm)	PdI	Zeta-potential (mV)	RA association efficiency (%)*
Blank-LNC	68 ± 2	0.059 ± 0.003	-4.8 ± 0.4	-
LNC RA	62 ± 2	0.064 ± 0.020	-7.4 ± 0.6	91.8 ± 2.9
LNC RA/DiR	64 ± 0	0.115 ± 0.004	--5.7 ± 0.6	94.4 ± 4.9
Mal-LNC RA/DiR	63 ± 0	0.080 ± 0.003	-5.2 ± 0.6	
TfRp-LNC RA/DiR	92 ± 2*	0.230 ± 0.022	-6.3 ± 1.7	

3.2 Impact of LNC on blood clotting time (or LNC hemocompatibility)

To comprehend the impact of the LNC when in contact with blood, it is important to verify if these were not capable of causing an adverse complement activation reaction. Thus, the clotting times of the recalcified plasma in the presence of differing stages of the LNC was verified. The wells with plain water should be seen as the upper limit for the clotting time, while the glass coverslips should be the lower limit, as it is a potent activator of the coagulation cascade [26]. As can be seen in Figure 1, the clotting times in presence of LNC were the same than the negative control (water) regardless of the formulation and had significantly lower values compared to the glass wells, suggesting that the LNC do not prematurely activate the coagulation cascade when in contact with blood.

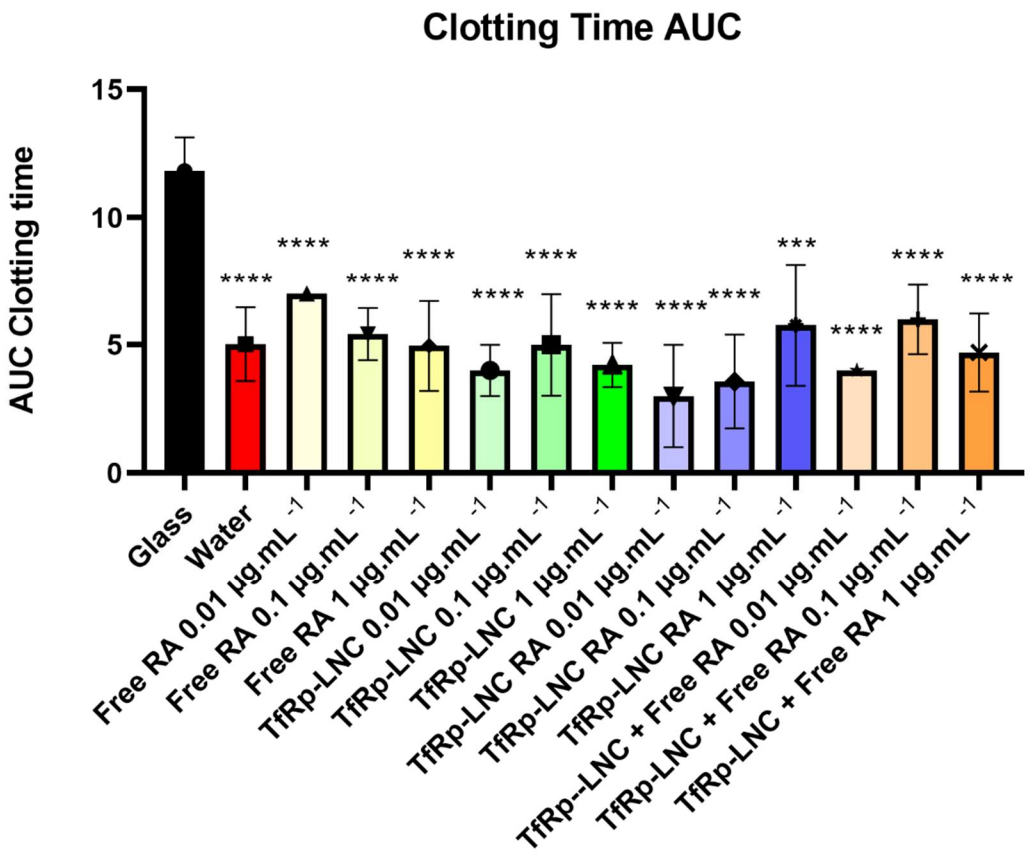


Figure 1. Impact of LNC on the kinetics of the clotting time of recalcified plasma in the presence of RA, blank-LNC, TfRp-LNC RA, and the physical mixture of LNC and free RA, shown as area under the curve. All samples (N=5, n=3) were placed in a 96-well non-tissue culture plate.

3.3 Transport of LNC across an *in vitro* model of human nasal epithelium

In order to study LNC transport across an in vitro model of the human nasal epithelium, LNC impact on cell viability was evaluated. The timing of the incubation was selected to simulate the acute and subacute exposure of the LNC to the nasal cavity cells. The range of concentrations was based on our previous work, in a concentration range where RA had an impact on neural stem cell differentiation and on pro-inflammatory microglia suppression [23]. After 15 min, no toxicity was observed, while a small but significant decrease of cell metabolic activity was seen after 1h of incubation for blank-LNC (Figure 2).

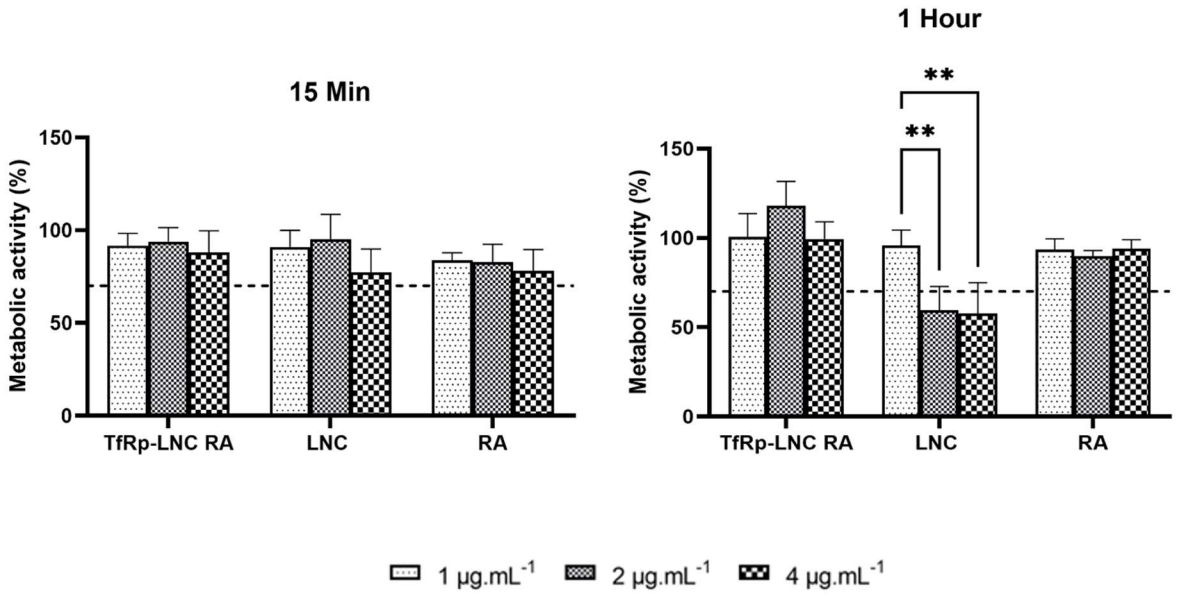


Figure 2 Metabolic activity of human nasal epithelia cells (RPMI 2650) at 15 minutes and 1-hour post-exposure. The concentration of RA was calculated based upon dilution factors of LNC. Results from *n*=3 independent experiments (five replicates per condition); Mean +/- SD *p<0.05.

As the average half-life residence time for mucoadhesive formulations is around 45 minutes, these results suggest that the LNC will be safe for intranasal administration, as the toxicity only becomes apparent after an hour [27]. LNC have already been used as intranasal delivery systems, and they have shown that they are safe and tolerable for administration and can promote the accumulation of bioactive therapeutics at the CNS [28].

Bypassing the BBB through the nasal epithelium is one of the candidate pathways to improve the permeability of compounds towards the CNS. Compared to the intravenous route, or direct delivery to the CNS via intrathecal or convection enhanced delivery, it provides a noninvasive alternative that aims to bypass the BBB rather than attempting to penetrate it directly. The same considerations when designing the system for intravenous should be considered for the intranasal route, as LNC is composed mostly of high concentrations of lipids and surfactants, higher dilutions should be prioritized, ensuring the drug loading is sufficient to allow bioactive concentrations.

Then, we studied the transport of LNC across a model of the human nasal epithelium using an already established in vitro model. RPMI 2650 cells offer an approximation to the behavior of the epithelial cells of the nasal cavity of humans, although it will not give an exact approximation of the nose-to-brain route [29, 30]. Through the liquid-liquid interface that is established the results from this model offer a tentative prediction of the behavior of the formulation when in contact with the nasal epithelium. The integrity of the model tight junctions was validated by assessing the TEER throughout the experiment.

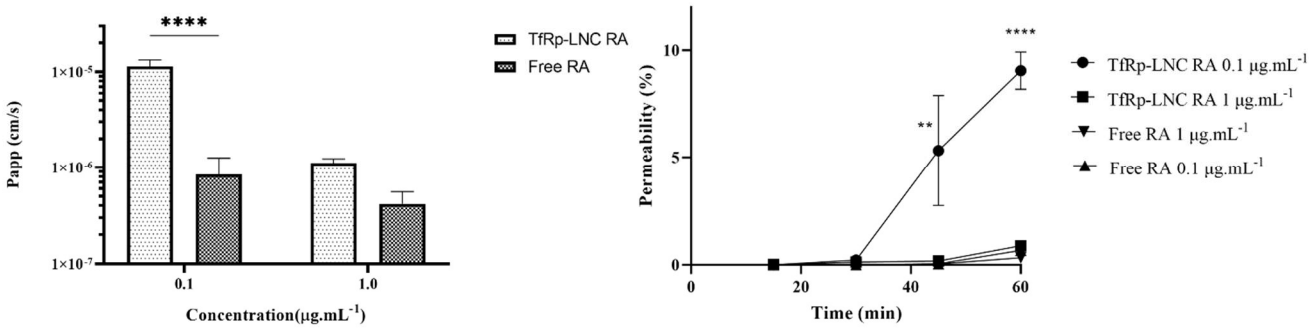


Figure 3 Permeability of the LNC compared to the free RA in a nasal epithelium model. (A) shows the apparent permeability (Papp) at one hour while (B) shows the kinetics of the permeability through an hour. Results from N=3 triplicates.

The permeability shows a striking similarity to previous results, that showed a higher permeability coefficient of the lowest dose formulations, suggesting some saturation occurring at the cellular level or regarding the levels of RA [23]. Despite the nasal epithelium primarily bypassing the BBB, some of the particles can enter the bloodstream by differing pathways are presented before. As the values here are similar to the expected values by direct permeability through the BBB, both can be seen as comparable routes of administration towards CNS delivery.

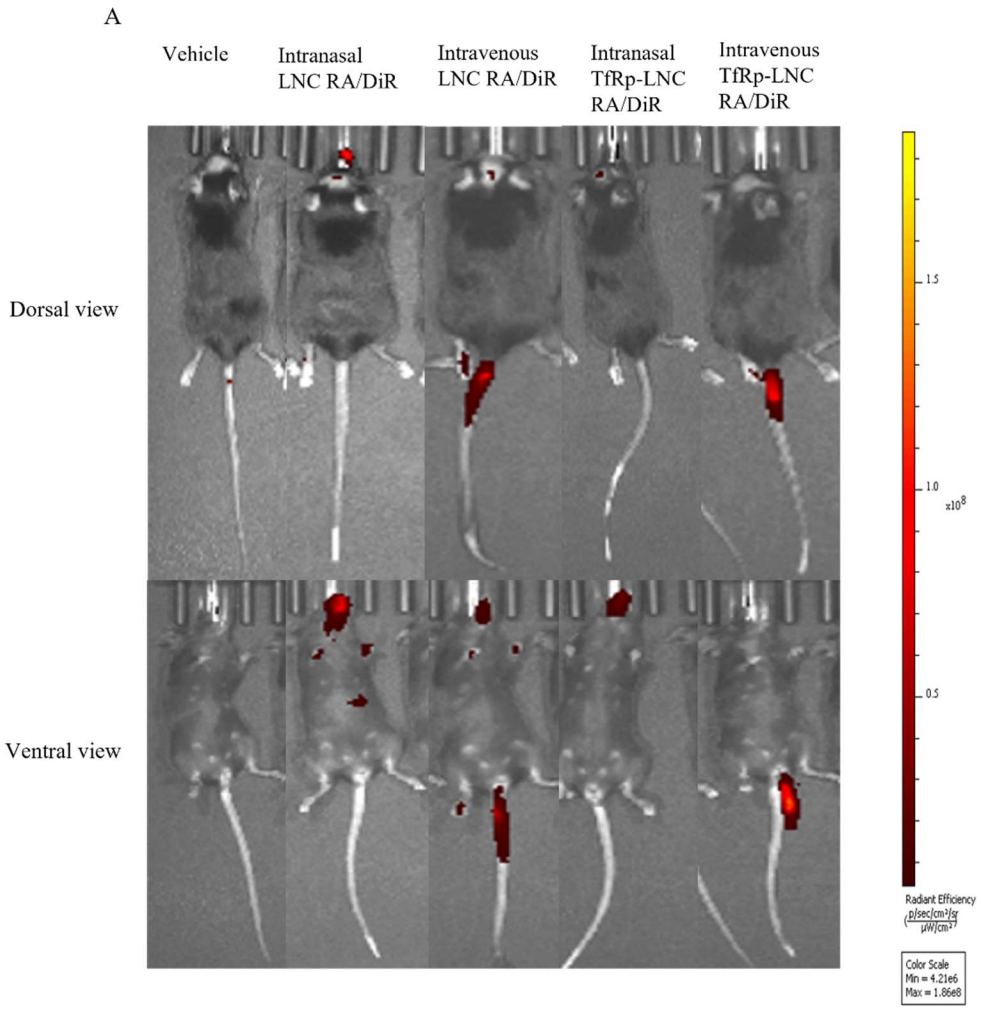
3.3 Biodistribution and safety profile of LNC

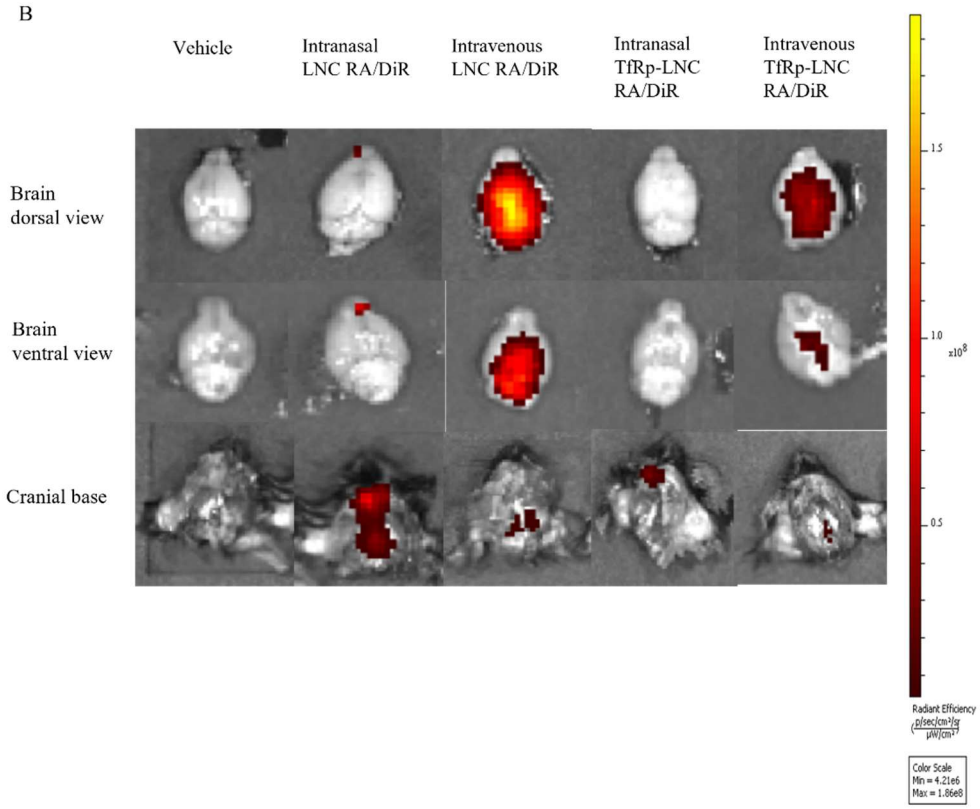
In order to understand the ability of the functionalized LNC to improve the pharmacokinetics of RA towards improved CNS permeability, it was important to fully elucidate the biodistribution in healthy animals. As the LNC were co-encapsulating DiR and RA, it allowed a monitoring of the safety profile of the LNC as well, to fully understand the interaction between the LNC and a living system. The accumulation of the LNC was then monitored using *in vivo* fluorescence imaging by monitoring the DiR signal in live animals, and then *ex vivo* after 7 days, 1 hour after administration.

From the results, it seemed that only intravenous formulations managed to reach the brain parenchyma as visible in the *ex vivo* brain images, while the intranasal had barely no differences to the vehicle, other than small signal at around the olfactory bulb (figure 4). On the cranial base, the reverse is observable – with intranasal being more pronounced than intravenous. Although the size of the LNCs should be within the optimal size for nasal delivery, it is closer to the upper limits of acceptable permeability rates, resulting in reduced interactions within the olfactory and trigeminal nerve endings. These suggest that through the intranasal route the particles might be washed away before they can reach a satisfactory concentration at the CNS, leading to it not being suitable to account for large accumulations within the CNS. Perhaps the most interesting result, is that the TfRp-cys functionalized LNC seemed to perform worse than its non-functionalized counterpart, potentially suggesting that the functionalization process is not able to aid in BBB penetration, but that the LNC due to their properties can still navigate it. Here, perhaps there could be an affect by altering the surface composition of the LNC, the protein corona that could be formed around the LNC might alter the way it is recognized by the immune system, or how it is able to interact with the endothelial cells of the BBB [21]. Assessing the interaction of serum proteins with the LNC could be a way to elucidate the behavior of the LNC. The increased size and PdI that the LNC also experience when functionalized with TfRp-cys might also contribute to the inability to correctly generate a receptor binding interaction between the peptide and the TfR. Similar findings have been shown before, where transferrin receptor targeting did not sufficiently improve the transcytosis process of the nanoparticles, but rather accumulated within the microvessels of the brain [31].

From the quantifiable data, the values accumulating at the CNS maintained steady throughout the 7 days, suggesting that the LNC would wash away before the next administration. Intranasal seemed to have more quantifiable signal, but it is likely due to the presence of LNC at the nostrils that dripped out during administration. In the *ex vivo* quantification it is seen that the intravenous route promotes a 1.2-fold increase compared to intranasal and the vehicle, which in CNS delivery terms can be considered a significant penetration, as when considering the CNS, permeability rates of as little as 1% have the potential to be beneficial [32]. The cranial base values confirm what was seen in the images, that intranasal administration seems to accumulate around the skull, but not reach deep within the parenchyma . This likely is occurring likely because most of the formulation drains out through the nasal sinuses rather than managing the transport through the nasal epithelium.

The organ distribution followed a predictable pattern, with the LNC following a more widespread distribution when administered via the intravenous route, while it preferentially accumulated in the gastrointestinal tract via the intranasal route [33]. The overall values of the organs are overlapping, suggesting that all groups got similar concentrations, excluding any possible differences regarding formulations.




B
Vehicle
Intranasal LNC RA/DiR
Intravenous LNC RA/DiR
Intranasal TfRp-LNC RA/DiR
Intravenous TfRp-LNC RA/DiR
Brain dorsal view
Brain ventral view
Cranial base
1.5
1.0
x10^8
0.5
Radiant Efficiency
Color Scale
Min = 4.21e6
Max = 1.86e8

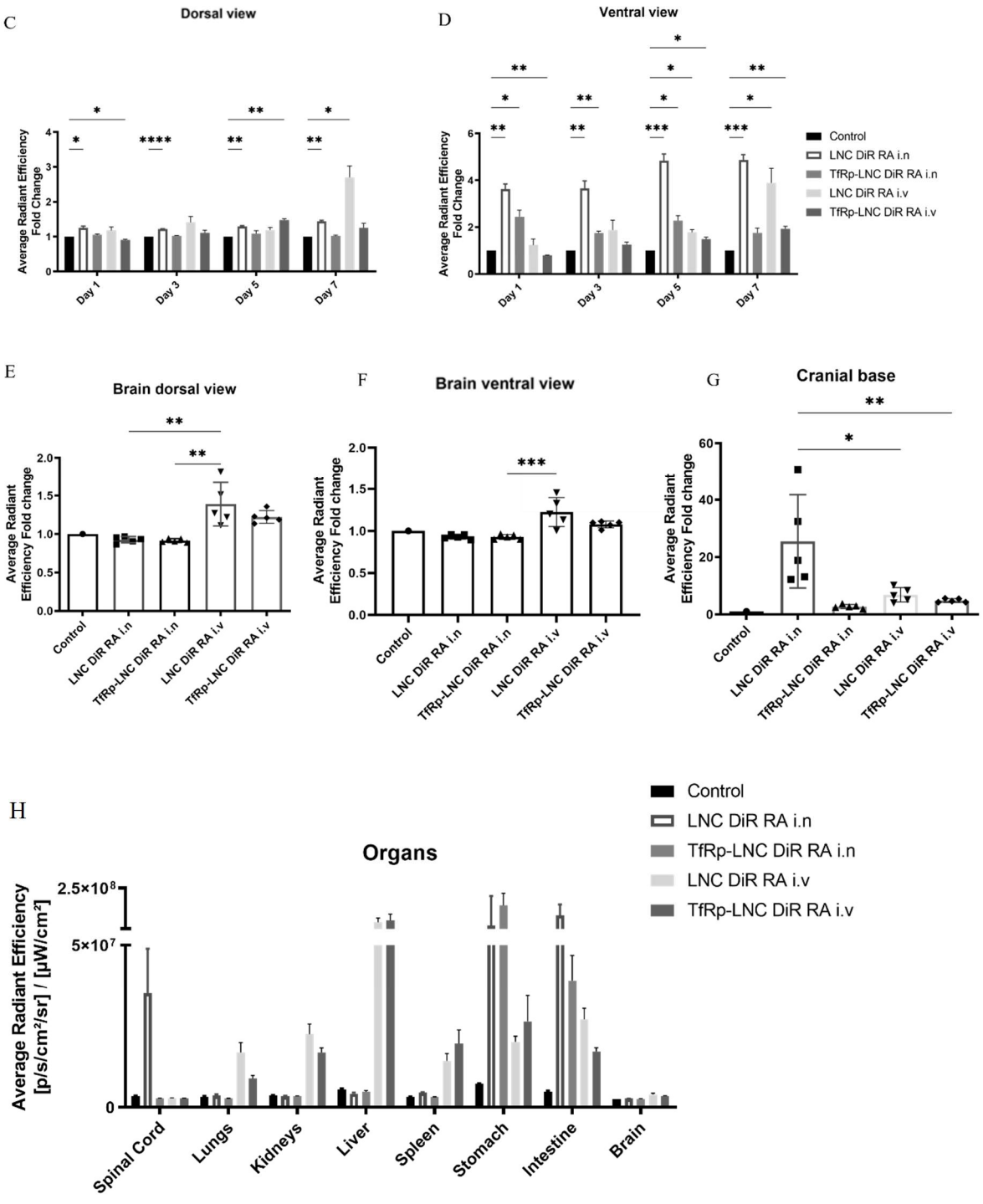

C
Dorsal view
Average Radiant Efficiency Fold Change
Day 1
Day 3
Day 5
Day 7
D
Ventral view
Average Radiant Efficiency Fold Change
Control
LNC DiR RA i.n
TfRp-LNC DiR RA i.n
LNC DiR RA i.v
TfRp-LNC DiR RA i.v
E
Brain dorsal view
Average Radiant Efficiency Fold change
F
Brain ventral view
Average Radiant Efficiency Fold change
G
Cranial base
Average Radiant Efficiency Fold change
H
Organs
Average Radiant Efficiency [p/s/cm²/sr] / [µW/cm²]
2.5×10⁸
5×10⁷
0
Spinal Cord
Lungs
Kidneys
Liver
Spleen
Stomach
Intestine
Brain

Figure 4 Biodistribution profile and organ accumulation of LNC RA/DiR at 7 days, post 1 hour of administration. A shows the full body scan of the mice after 1 hour of administration, while B shows the head region (brain and cranial base) immediately after sacrificing. C through H shows the measured radiant intensity quantified, presented as fold change normalized to the vehicle values, and the values from the organs presented as absolute radiant efficiency values. Results from n=2 experiments, with N=5 per condition. Mean +/- SEM *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

During this work it was chosen to dual encapsulate RA and DiR, the safety profile of the LNC was also assessed concomitantly with the biodistribution profile of the LNC. The weight was followed every day to validate if there were any signs of toxicity from the administration of the LNC, and post sacrifice blood was collected from the mice to check for the functions of key organs responsible for the clearance of the LNC. Figure 5 has the results from the safety profile of the LNC, and it was found that the TfRp-cys LNC were promoting some moderate weight loss through the 7 days (as high as 5%), which was confirmed by an increase of the liver enzymes (2.5-3 fold compared to the control and the remaining groups for the liver enzymes) and the creatinine values in the blood, which suggests towards some widespread toxicity caused by the peptide [34]. Here, there do not seem to be many reports in the literature suggesting toxicity by these kinds of peptides, but there is a study suggesting it can negatively interact with albumin [35]. As these results were unexpected and a bit paradoxical, it was opted to further run a longer-term assay to try to understand if this was a relevant finding or a clinically isolated happening.

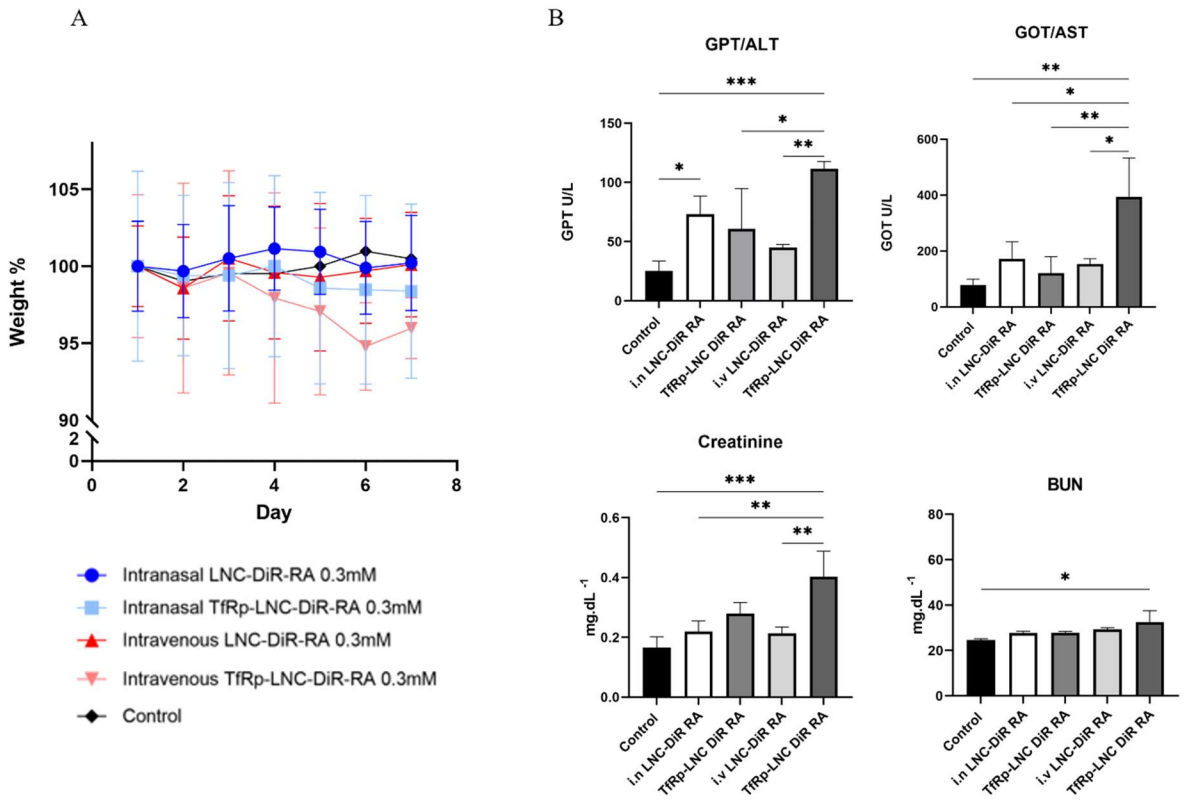
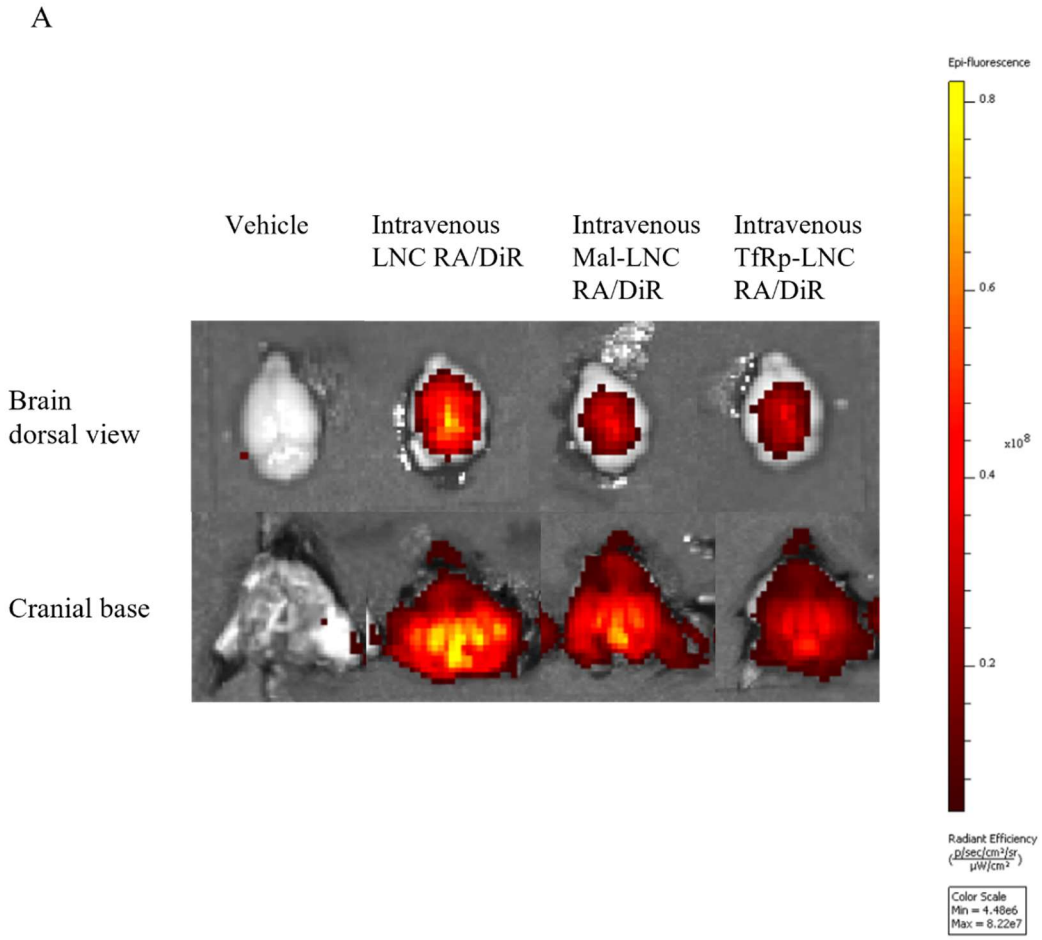


Figure 5 Safety profile of the LNC. A shows the weight of the mice followed during the assay, while B shows the biochemical parameters of key parameters related to the wellbeing of mice. Results from n=2, N=3 mice. Mean +/- SEM *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

Considering then the results of the previous biodistribution study, the intravenous route was selected as the main route to continue with the studies, but there still was the necessity to identify the issues with the TfRp-cys functionalized LNC, in order to understand both its lack of efficiency and its safety threat to the mice. Thus, a longer assay following the mice for two weeks was then selected. Indeed, in this assay it was found once again that TfRp-cys functionalized LNC performed worse than its non-functionalized counterparts, although all formulations improved the accumulation in the CNS by around 1.3-fold when compared to the control (figure 6), confirming then that the transferrin peptide was not able to reproduce its *in vitro* results in an *in vivo* model. These results are directly reproducible from the 7 days assay, with the fold changes being overlapping, suggesting towards the reproducibility of the results.

Although all the formulations are submitted to the same functionalization process, only when TfRp-cys is added the LNC tend to present with a higher size, and a less monodisperse population, potentially suggesting as a cause for its lack of effectiveness. As the non-functionalized versions preserve a smaller size, they might interact better with the BBB endothelia, crossing the barrier through an unspecific pathway rather than through receptor mediated transcytosis [36]. Notwithstanding, it should also be considered that transferrin is fairly ubiquitous throughout the body, and perhaps the LNC are being guided towards unwanted locations rather than accumulating preferentially in the CNS. Other potential causes could be the lack of acid-cleavable properties of the LNC-TfRp-cys covalent link, meaning the LNC could get recycled with the receptor back towards the brain capillaries [37], although more work would be required to confirm this statement.



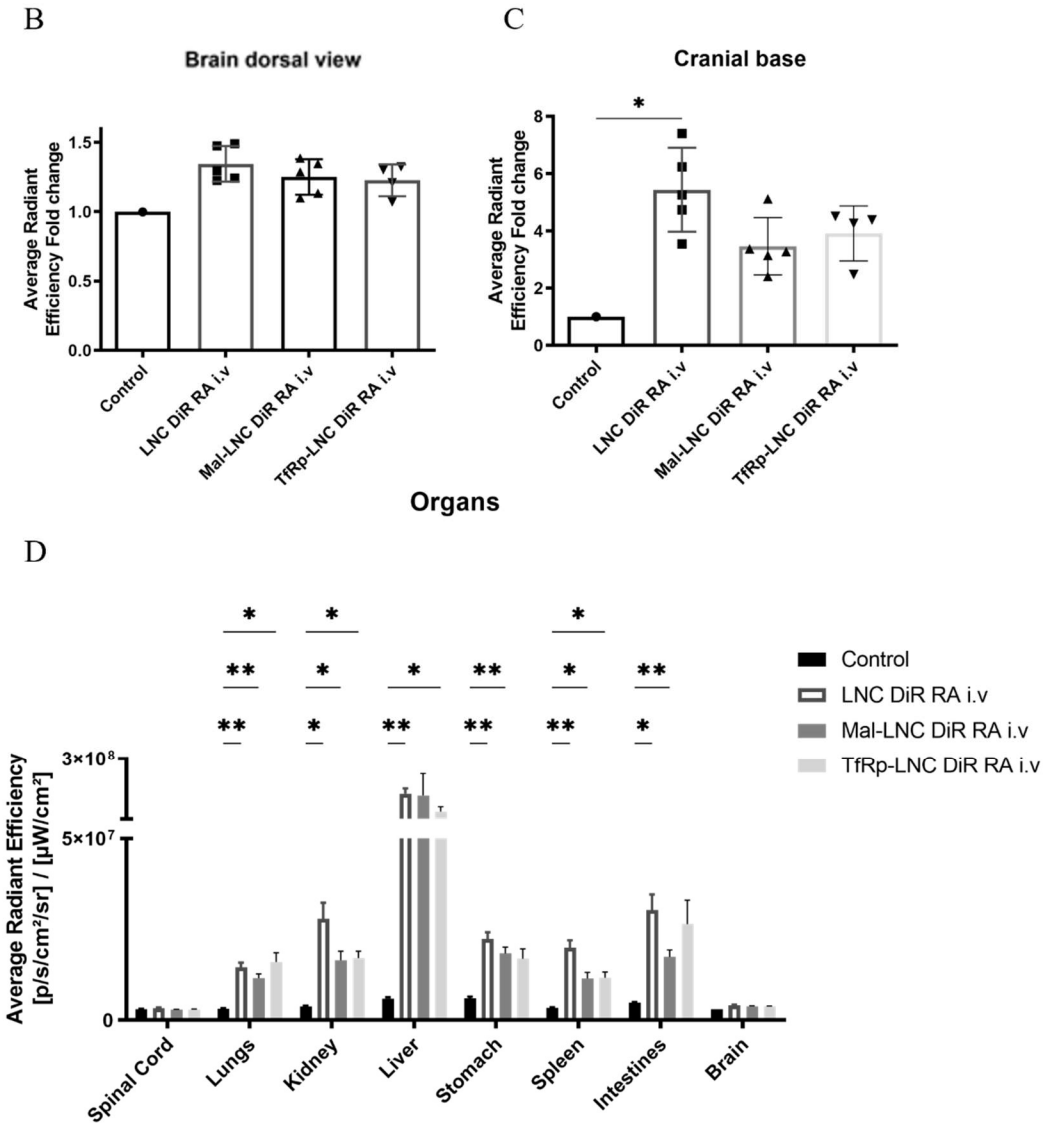


Figure 6 Biodistribution profile of LNC RA/DiR at 15 days, post 1 hour of administration. A shows the *ex vivo* imaging as intravenous full body scans tend to not show signal on the head region (data not shown), while B through D show the fold changes in fluorescence intensity of the formulations and the absolute values of the biodistribution to the organs. Results from n=1 experiment, with N=5 per condition. Mean +/- SEM *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

Regarding the safety profile of the LNC, a similar method was performed by monitoring the weight daily, and then collecting blood post sacrifice for biomarker analysis (figure 7). Through this experience, 1 mouse from the TfRp-LNC RA/DiR group died unexpectedly, with no apparent cause of death found on autopsy. Coupled with the elevated biochemical marker levels analyzed, it further points towards the lack of safety of TfRp-cys functionalized systems. Thus, further work should be carried out on systems that are formulated through this functionalization strategy with this particular peptide, to try to understand the potential lack of safety observed.

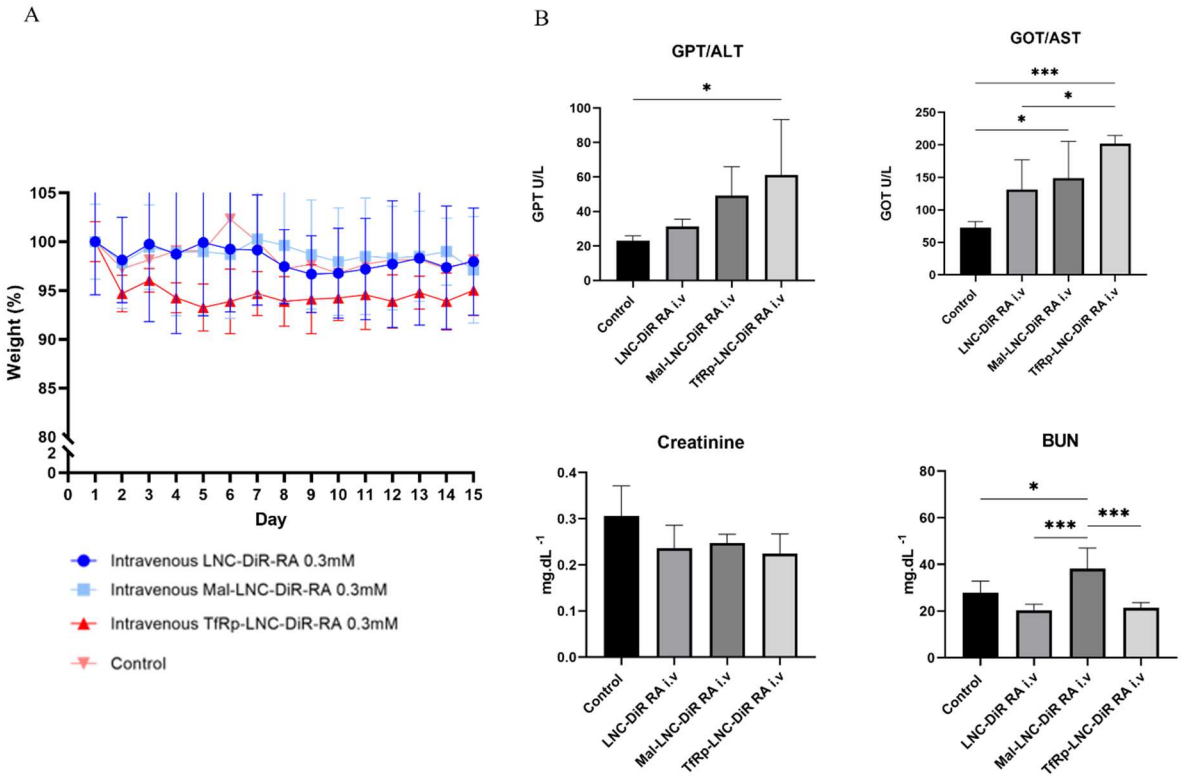


Figure 7 Safety profile of the LNC following a 15-day treatment regimen. A shows the weight of the mice followed during the assay, while B shows the biochemical parameters of key parameters related to the wellbeing of mice. Results from n=1, N=5 mice. Mean +/- SEM *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

Following the data that was acquired from these assays, the plain LNC RA was selected to be evaluated in the EAE model, to try to prevent any unwarranted effects from occurring that could worsen the condition.

3.4 LNC RA impact on EAE after intravenous administration

The EAE model is considered one of the gold standards to test novel compounds in MS and to study the physiopathology of the disease. It is characterized by widespread CNS inflammation coupled with focal demyelination lesions, mostly occurring at the spinal cord [38]. The cerebral and cortex zones are primarily unaffected by this disease phenotype.

In this design, it was chosen to only begin treatment after 10 days from the induction of the disease phenotype, to allow the early stages of the disease to occur in a natural form, and to then validate the potential of RA to restore the lesion locations. After 10 days, mice that were immunized with the induction cocktail started presenting signs of the disease, and the treatment was initiated with administrations every other day. RA by itself was not administered due to the lack of any feasible solubilization method that allowed for a safe administration of RA. As the LNC act as a surfactant to shield RA from premature degradation and improve its solubility, the use of any surfactants would make the use of the LNC redundant.

Compared to the vehicle (Figure 6), neither LNC RA and blank-LNC, although there were no statistically significant differences in the clinical scoring of the mice, treatment with the LNC seemed to reduce the maximum score to which the animals progressed and had some role delaying the disease onset. The area under the curve (AUC) which is suggestive of disease severity was reduced in animals' treatment with LNC RA compared to the vehicle by 1.7-fold (Table 2), potentially suggesting some ameliorating features of the LNC in MS.

Table 2. Clinical scoring and disease progression of EAE mice after treatment with LNC RA and LNC. Results from n=1, N=10. Mean ± SEM.*p<0.05.

	DISEASE INCIDENCE	MEAN OF MAXIMUM SCORE	MEDIAN OF MAXIMUM SCORE	MEAN DAY OF ONSET	AUC
EAE-VEHICLE	10/10	3,6 ± 0,1	2,6 ± 0,3	12,8 ± 0,4	49,42 ± 1,90
EAE-LNC RA	10/10	2,8 ± 0,3	1,5 ± 0,3 *	14,0 ± 0,8	29,61 ± 4,19 *
EAE-LNC	9/10	2,9 ± 0,4	1,8 ± 0,3	12,9 ± 0,6	36,05 ± 6,07

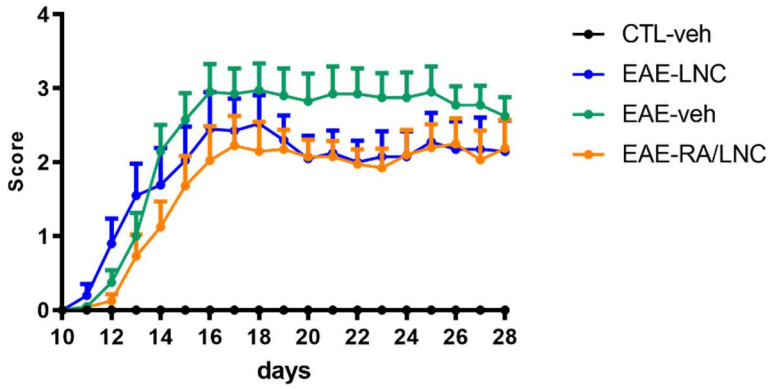


Figure 8 Clinical scoring progression of mice after EAE induction. N=9-10 animals, +/- SEM *p<0.05

When LNC RA was exposed to microglia *in vitro,* it was observed a significant reduction in the pro-inflammatory markers that are expected when the microglia undergo a phenotypic shift towards an aggressive, pro-inflammatory state. To understand if this was replicable *in vivo,* sections of the spinal cord from affected mice were analyzed for their expression of key markers, to validate if the system was able to significantly control the inflammation at the lesion sites.

Here, LNC RA did not seem to be capable of reproducing their results *in vitro,* and no substantial decrease in the inflammatory markers mRNA was found throughout (Figure 8). Throughout the expression of MIP1α, ATF3 and TNFα, no statistically significant differences were found between the EAE mice, and the mice treated with either the blank-LNC or the LNC

RA. The comparable decreases visible with both LNC and LNC RA is something that was seen before, with the LNC by themselves carrying a modest effect *in vivo [39].* Although in this case, the combination of LNC and RA did not seem to significantly improve the therapeutic outcome, it does present some leeway for the LNC to be further exploited with alternative therapeutic options. Paradoxically, when exposed to LNC RA there seemed to be a slight increase in TNFα. The rationale behind this increase is something that is not understood and would require further investigation to verify, although the standard deviation observed suggests it might have been an isolated finding. Although there was a slight increase in IFNγ, which is a vital anti-inflammatory marker produced by cells and has been linked with an improved outcome in MS and is indeed an approved therapy in certain cases [40], it was not statistically significant. The MOG values which are related towards the presence of myelin in spinal cord demonstrate that demyelination occurred throughout, but the modest increase compared with the vehicle and the LNC and LNC RA did not show statistical significance nor clinical significance.

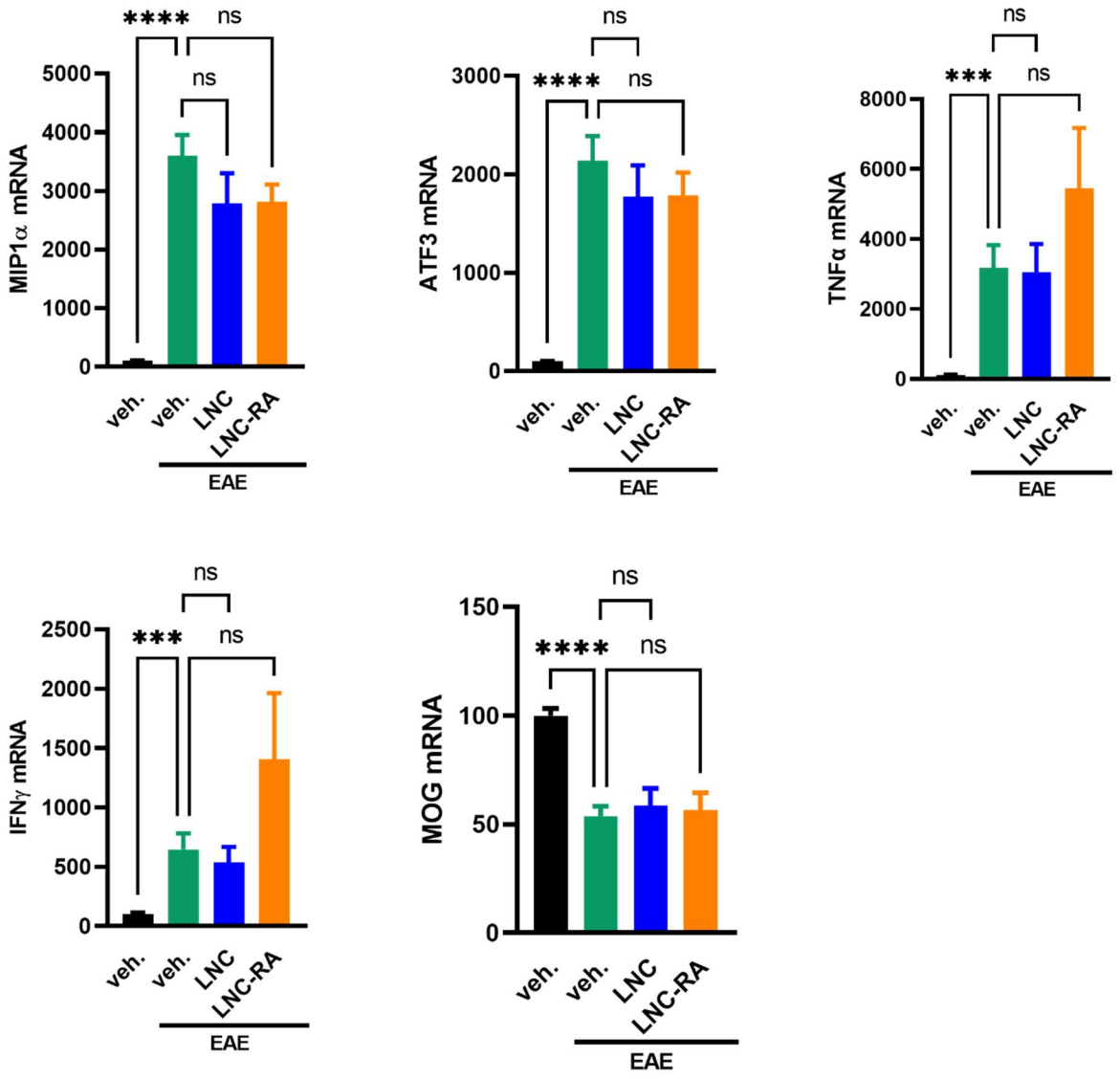


Figure 9 PCR analysis of mRNA of certain inflammatory markers in sections of the spinal cord of EAE mice. Results from N=9-10 mice, Mean +/- SEM *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

Regarding the production of myelin, analysis by the fluoromyelin (Figure 10) method showed that the LNC RA was able to significantly reduce the rate of demyelination compared to mice that were given the vehicle only. Both the images and the quantification suggest that on the

LNC RA group the area of demyelination is markedly reduced and the overall structure of the whitter matter is more organized, as has been seen in other works. This might be intrinsically related to the improved expression of IFNγ which is a key mediator of inflammation resolution in the scope of MS. This result suggests that LNC RA could be capable of restoring demyelinating lesions that are seen in MS patients', and this lines up with the observed improvement to the maximum score observed in the mice as well as the reduced AUC.

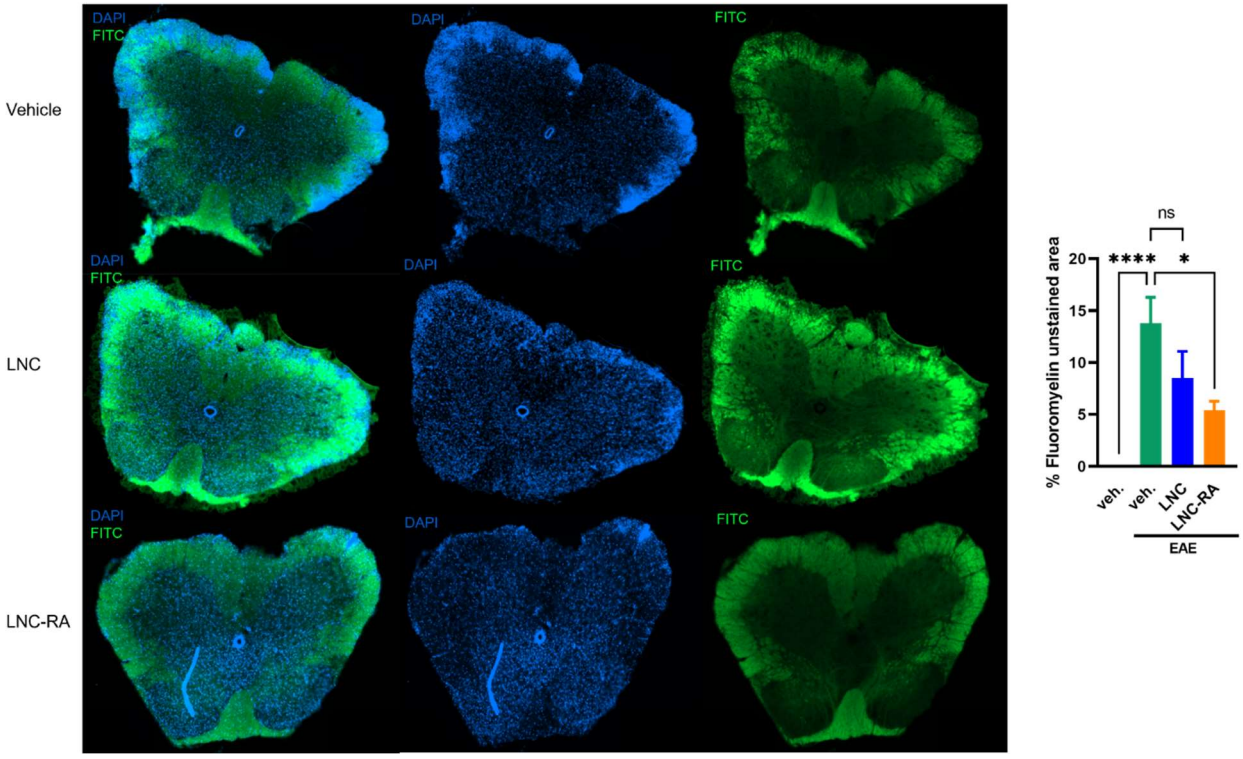


Figure 10 Fluoromyelin analysis of spinal cord sections of mice. On the left the overlapping image, where the blue areas with high number of cell nuclei show demyelinating areas Blue=DAPI, Green=Fluoromyelin. Results from N=8-10 mice, Mean +/- SEM *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

To understand the impact that the LNC had on other cellular player of the CNS, a staining for GFAP and IBA1 was performed (Figure 11). GFAP, an intermediate filament-III protein, is directly correlated with astrocytic activation in neuroinflammation offers , and high values of this marker suggest that there is an ongoing astrocytic activation at the CNS of mice [41]. Iba1 on the other hand is a microglia specific marker, that identifies the presence of microglia infiltrates.

Our results showed that the induction of EAE triggered an activation of both the astrocytes and microglia at the spinal cord, but the LNC RA were not able to decrease this gliosis. This suggests that the treatment option is not capable of preventing the establishment of a glial scar in the affected spinal cord, which could symbolize an inability of LNC RA to act in advanced stages of MS where demyelinating injuries are populated mainly by reactive astrocytes and microglia [42]. This suggests towards the hypothesis that RA primarily should be considered as an early treatment of MS when the demyelination is not overwhelming, and where stimulation of OPCs mobilization and differentiation might be key.

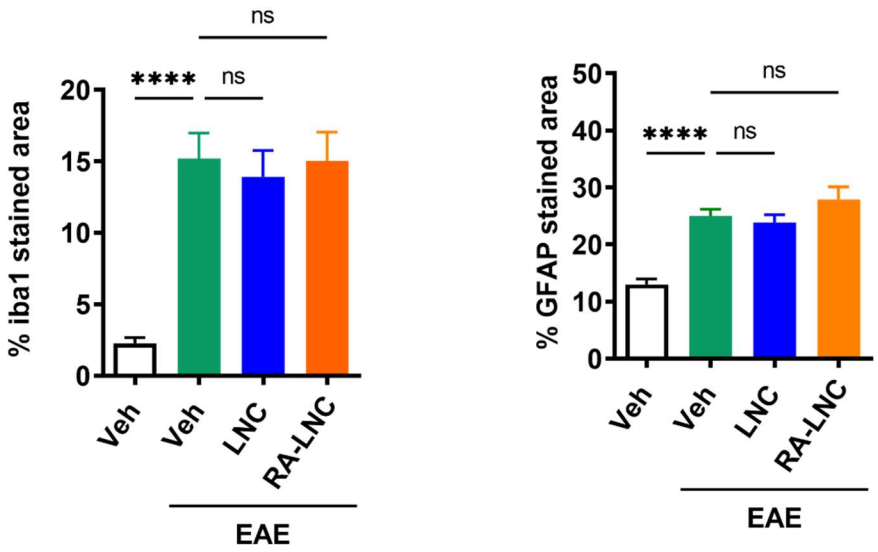


Figure 11 GFAP and IBA1 expression in spinal cord sections of mice. Results from N=8-10 mice, Mean +/- SEM *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

4. Conclusions and future perspectives

This worked carried on the development of a BBB-targeted LNC-based nanosystem, where its impact on a nasal epithelium and in an *in vivo* model. The LNC showed that it was safe for intranasal administration, and it was able to navigate the nasal epithelium compared to just the free RA. When comparing the administration routes, intravenous showed a higher quantity of accumulation in the CNS compared to the intranasal route. Perhaps most surprisingly, the TfRp-cys peptide placed at the shell of the LNC revealed to not be effective at all in improving

the targeting towards the BBB, and even caused some marked toxicity in its groups, leading to its discontinuation as a formulation strategy. Nonetheless, the formulation was tested in an EAE model to mimic the development of MS *in vivo.* Results showed that the LNC were not able to significantly suppress the inflammatory environment at the spinal cord of mice but did have a modest increase in IFNγ. Perhaps the most important result was the 2.5-fold reduction in fluoromyelin unstained area at the spinal cord of mice compared to the vehicle, suggesting that the LNC RA are able reduce the aggressive demyelinating behavior seen in lesion sites of MS.

These results show that the system still requires further optimization, but it carries some promise as a treatment option for MS. In the future, co-therapies with more potent anti-inflammatories could elevate this nanosystem to new heights, and present itself as a therapy for MS.

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CHAPTER 5 – CONCLUSIONS AND FUTURE PERSPECTIVES

1. General discussion and conclusions

The rationale design and the use of bioengineering as innovative means to advance pharmaceutical development of novel therapies has been an emerging field. Ultimately, its goal is reaching personalized, effective therapy to target all ailments. Indeed, through the interconnection of the many fields in the broad world of the development of new drugs, science as a whole progress towards an overall improvement of therapeutic outcomes in numerous areas and ensures that unmet clinical needs are tackled adequately. For the central nervous system (CNS), this is particularly paramount, as it is an area where disease incidence is steadily rising, and therapeutic options have not advanced as efficiently as in other areas.

Therefore, throughout this thesis, the rationale of the work followed a compromise between the areas of nanotechnology and blood-brain barrier (BBB) targeting to attempt to end up establishing a successful candidate for the delivery of therapeutics aimed at neurodegenerative disorders.

As was discussed throughout the work, the lipid nanocapsules (LNC) which were widely used in literature for their ability to encapsulate lipophilic drugs were used as a vehicle to encapsulate a highly unstable molecule with interesting therapeutic potential, retinoic acid (RA). These LNC were co-encapsulated with super paramagnetic iron oxide nanoparticles (SPIONs) and were then submitted to a post-manufacture process to incorporate a transferrin-receptor binding peptide (TfRp) on their surface to theoretically improve their BBB targeting affinity.

The LNC core system that was chosen as the primary carrier was responsible for acting as a solubilizer for RA. This is due to their innate structure similar to a lipoprotein responsible for shuttling cholesterol and other lipids around to body, and the ability to prevent RA premature degradation within the vehicle and to ensure it is not rapidly cleared out by the body by the metabolic pathways available. The LNC system in itself would only primarily interact with the BBB endothelium through passive diffusion, due to their constitution being primarily lipidic,

which would theoretically otherwise not address the limiting behavior of the BBB and penetrate it satisfactorily. To better the odds of achieving satisfactory results, the LNC should be developed with a nominal size that is said to improve the penetration of nanoparticles through the BBB (below 100nm is ideal, with 100-200nm being acceptable) [1, 2]. This size range is not only beneficial for BBB interaction, but it also creates an unfavorable environment for uptake by the reticuloendothelial system (coupled with the polyethylene glycol coating of the LNC), improving their circulation time [3]. The developed functionalized and unfunctionalized LNC had a mean size of 70 and 109 nm respectively, both presenting as a slightly negative (-3 to -7 mV) zeta-potential. Due to the innate properties of the LNC, establishing a highly lipophilic core stabilized by the presence of external amphiphilic surfactants, RA had high affinity for the LNC, resulting in high encapsulation rates of around 90%. The surface functionalization did not compromise the association of RA, and the chemical transacylation reaction established between the surfactant and a maleimide-modified polyethylene glycol to allow for thiol linkage with a cysteine modified peptide was fairly stable. Due to the low drug loading and the large quantity of lipids, the safety profile of the LNC was an important attribute to assess, and the LNC showed that they were able be safe and tolerable in acute and subacute timepoints, maintaining viability rates above 90% at active concentration windows where RA is effective. Then, it was validated that the surface functionalization process was able to significantly increase the accumulation of the LNC, both qualitatively and quantitatively through confocal microscopy and flow cytometry measurements by 2-3-fold in endothelial cells of the BBB that are commonly used as predictors of BBB behavior *in vitro*. This was further demonstrated when the receptor was fully saturated with exposure to the same peptide before treatment with the LNC, to symbolize that the cells uptake the LNC primarily through the transferrin receptor pathway rather than passive micropinocytosis or diffusion. As the transferrin receptor is an overexpressed receptor by the endothelial cells of the BBB, its behavior has been well studied. Transferrin-targeted uptake is internalized following clathrin dependent mechanisms, meaning that the complex of the receptor and the LNC is “enveloped” by the membrane in a clathrin-coated pit that follows the early endosome pathway, with the receptor protein being recycled back to the surface [4, 5]. However, as this process takes time, it is possible to saturate the receptor pathway [6]. The LNC in itself are able to “escape” the endo-lysosomal pathway with as little as 10% of the payload being prematurely released, being able to be cycled on the other side of the endothelial cells, allowing them to release their payload to the target cells only when the cell membranes fuse with each other [7]. To further validate these results, a defined

monolayer based on the same cells was created capable of mimicking BBB properties, and it was found that the presence of the peptide improved the permeability of the LNC by 5-fold compared to unfunctionalized controls. As LNC are very similar to lipoproteins, it was important to clearly establish the difference between passive uptake and active uptake, lest the functionalized not be working as intended in the surface of the LNC. The rationale behind the assessed differences then points towards the LNC being able to recognize the transferrin receptor and exploiting it as a method of internalization that augments the ascertained rates obtained when only a mock functionalization process is done, and TfRp is not expressed at the surface of the LNC.

As RA had been previously demonstrated to have an effect in both inflammation and the stimulation of the differentiation of neural stem cells and cells from the oligodendrocyte lineage, it was important to validate that this effect was carried over when it was solubilized within the core of an LNC. Thus, the LNC were assessed for their ability to interact with oligodendrocyte progenitor cells at differing periods of differentiation and microglia through confocal microscopy, and it was seen that the particles were able to be interiorized by the target cells. Due to RA target receptors being intranuclear [6] and RA not having a defined pathway to get inside the cells, as it is done only when it is travelling linked to the retinoid-binding protein, demonstrating that the LNC can shuttle RA deep within the cell is paramount towards the outcome of the project [8]. When exposed to LPS, microglia undergo differentiation into a reactive, pro-inflammatory phenotype, undergoing rapid proliferation and adapting a more ameboid state that stimulates production of pro-inflammatory cytokines and reactive oxygen and nitrogen species capable of promoting tissue damage, namely to myelin at the CNS. Within the scope of this thesis, it was found that when RA is present in activated microglial cells, it is able to not only suppress the pro-inflammatory cytokines in about 50-70%, but also to stimulate the production of anti-inflammatory cytokines (IL-10), suggesting an innate ability to control the inflammatory environment present at the CNS. When this environment is controlled, there are fewer lesion sites, and there is less of a necessity for the development of reactive astrocytes to create glial scars, resulting in permanent loss of damage [9].

However, merely controlling inflammation is not enough to halt the ongoing myelin destruction that occurs bit by bit, and it is important to attempt to promote the innate machinery that is responsible for remyelination to be more efficient within MS. As people age and in MS, remyelination seems to falter or occur in reduces rates, and there is currently no therapeutic

option that manages to significantly address this axel [10]. As RA is able to differentiate neural stem cells through direct retinoid-X receptor stimulation, it was tested if it was able to induce the differentiation of oligodendrocyte progenitor cells, increasing the rate of differentiation compared to the lack of exposure to it. Although in this work there was no demyelinating damage present during the assays, it was found that RA when present in LNC was able to stimulate more mature and healthier oligodendrocytes when compared to no treatment at all, or even the free version of RA. This likely occurs because when it is solubilized within the LNC, it is able to interact with the cells and stimulate its nuclear receptors, while in the free version it likely precipitates due to its high hydrophobicity.

Having validated the developed nanosystem in its entirety in *in vitro* models, the focus shifted towards demonstrating that the effect is able to be translatable to more complex systems such as living organisms. To first validate that the system was suitable for administration, an assay assessing its ability to coagulate blood was done in blood from healthy donors. No complement activation was found and neither the LNC nor the free version of RA activated the coagulating chain more than the positive controls.

When the nanosystem was administered in healthy mice to assess its biodistribution and safety profile, comparing the potential of the TfRp-cys to improve brain delivery *in vivo* compared to fully bypassing the BBB through the intranasal route, perhaps a surprising result was found where it did not improve the accumulation at all, but actually promote a more sickly state of the mice, expressed by increase in liver enzymes and worsening of the kidney function of mice. Although this result was surprising and not predicted, it might be related to the ubiquity of transferrin in the whole body, resulting in more off-target effects than the unfunctionalized system [11]. It should also be considered that this LNC system does not have a cleavable location at the peptide, which might cause it to get recycled back to the brain capillaries if it is not able to dissociate in the early endosome. Nonetheless, the unfunctionalized LNC seemed to outperform the intranasal route, perhaps due to its high affinity for cellular membranes and achieving satisfactory brain delivery rates that allowed the work to continue.

To finalize, the selected optimal LNC was then tested in an MS-replicating model, experimental autoimmune encephalomyelitis where it showed that both the unloaded LNC and the RA loaded LNC was able to reduce the disease severity, slightly delay the disease onset and improve the clinical scoring of mice. Despite this, when considering the inflammatory environment at the

spinal cord, the improvements compared to the vehicle were modest, and held no statistical significance, suggesting a lack of translation from the *in vitro* findings to the *in vivo* panel. However, when considering the fluoromyelin stained area, the LNC showed a clear reduction (2.5-fold) compared to the vehicle, suggesting that RA was able to significantly improve the demyelinating environment at the spinal cord, suggesting that it is a viable candidate to restore lesion sites in MS. Nonetheless, the LNC RA did not seem to show an impact on the gliosis that occurs in lesions sites of MS, suggesting that it is not a viable late stage treatment option, where most lesion sites are covered by glial scars.

All in all, this nanosystem started as a complex structure that had to be made progressively simpler due to certain parts not working as intended, but nonetheless it showed some promise in the most important area of the work, which was restoration of damaged myelin in the CNS. Much like everything in science, it is required to start thinking ahead of optimizations to carry this nanosystem towards clinical approval.

2. Future perspectives

Lipid nanocapsules have proven throughout this work to be an effective carrier to deliver therapeutics towards the CNS, with the seemingly paradoxical find of having by themselves an innate action in impacting the course of the disease. Although the ideation of this work and the goal in itself facing some road bumps through the inability to fully harness magnetic targeting and the peptide functionalization being more harmful than beneficial when administered to animals, the future work that can be extrapolated and perfected from this work carries an immense weight. The continuous opportunity to perfect the nanosystem and to fully understand some results does not allow future opportunities and applications to falter.

Although CNS disorders still prove a worthy foe for most researchers around the world, the little dents that works like this carry, further guide the world towards therapeutic options that might prove more beneficial. The functionalization process could and should be perfected by the addition of acid cleavable properties to disassociate the LNC from the peptide, to prevent it being recycled towards the surface.

A way to further optimize this work would be to head back to the drawing board regarding the active target moiety and select one that is more specific towards the brain, even with a little less affinity than transferrin, and seeing if it would be possible to decrease the off-target effects, and actually

translate the *in vitro* results towards the *in vivo* models, and perhaps towards a clinical application. Receptors such as low-density lipoprotein receptor, insulin receptor might be less ubiquitous than transferrin and be viable candidates for delivery [4]. Tentative work with cell penetrating peptides with LNC has already been carried out and could prove to be a viable option to carry on the work [12-14]. Ideally, this work could be complemented by fully characterizing the presence of the functionalized peptide at the surface of the LNC and carrying out a study for the binding affinity of the receptor to fully optimize the functionalization process.

The impact in the differentiation of oligodendrocytes was an important hallmark for the validation of the system *in vitro*, but this study was done in progenitor cells removed from otherwise healthy mice pups. There was no assessment of the ability to induce synthesis of new myelin after demyelinating damage *in vitro*, thus not being able to draw conclusions from the potential of RA to act in an hostile environment that is a lesion site *in vivo.*

Although RA was the chosen candidate molecule, synthetic retinoids have chosen to be viable candidates for delivery, with better receptor binding properties, and more affinity for the intranuclear receptors. 9-Cis-RA or betaroxetene have been gaining some traction as candidates for therapeutic options, with betaroxetene having reached clinical trials as of recently [15]. Furthermore, the opportunity to develop dual combination schemes with LNC could also open an opportunity window that should not be put to the side.

To conclude, it should also be considered that this approach could be translated to a myriad of other CNS pathologies such as Alzheimer's, Parkinson's, or even glioblastoma. This work demonstrated the versatility of the LNC as a system in itself, and as hereby described, there are numerous suggestions that could be considered for upcoming researchers to elevate this work to new heights, towards the progression of science for CNS pathologies.

3. References

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