Delivery of microRNAs as a remyelination strategy in Multiple Sclerosis.

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Cover Image: Abstract painting representing a confocal image of a cerebellar organotypic culture. Myelin is represented in red, axons in green, myelin-axon colocalization in yellow and nucleus staining in blue. Iñaki Osorio Querejeta©.

A todas las personas que han confiado y creído en mí

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Doce menos diez de la mañana. Estación del Norte. Yo esperaba con cierto nerviosismo al tren que nos llevaría hasta Barcelona. Tres días en los que aprenderíamos nociones básicas para la generación de un modelo animal que a la postre serían fundamentales para esta tesis. La hora de salida del tren se acercaba, y yo seguía esperando a que un tal Matías, el cual me habían presentado en una ocasión, llegara para poder subirnos a nuestro vagón, puesto que yo no tenía los billetes. Finalmente, tras un pequeño sprint, subimos al tren y este partió.

Aquí comenzaba mi andadura en el grupo de esclerosis múltiple de Biodonosti. Pero he de reconocer que desde bien pequeño muchos factores me han ido encaminado hacia lo que hoy plasmo en estas páginas. Mi ama, bióloga recientemente retirada, me enseñó de manera inconsciente, o así lo percibo yo por lo menos, el amor por la biología. Pero no estaba sola en esta tarea, Santi, mi profesor de biología en el colegio, y puedo decir el mejor profesor que he tenido, se encargó de poner la puntillita para que decidiera estudiar biología. Sin embargo, esto quizás no hubiera tenido lugar si mi aita no me hubiera transmitido la tranquilidad, seguridad y confianza para poder hacer lo que me gustaba.

El tren ya está llegando a su próxima estación, donde tengo que apearme. Ha sido un viaje duro, pero en el que no podía haber estado mejor acompañado tanto personal como profesionalmente. Se me hace difícil por donde empezar; mucha gente que ha resultado muy importante para mi.

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Tampoco estaría de más si dijera que ha sido un verdadero placer aprender y disfrutar del que considero mi hermano argentino, Matías, con el cual tuve la suerte de poder montarme en ese tren. O que la atención, cariño y consejos de Haritz han sido una autentica maravilla y un placer. No quedaría mal si mencionara las amenas charlas con

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Pero no puedo limitarme únicamente a la UEM, en este trabajo han participado muchísimas personas, que si bien quizás sus nombres no aparecen reflejados, una parte de ellos se encuentra entre estas líneas.

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Hace años que nos conocimos en la facultad de Biología. Desde entonces hemos seguido un camino similar, en el cual el uno sin el otro no hubiéramos sido capaces de superar las dificultades con tanta facilidad. No soy capaz de imaginarme la tesis sin tu

apoyo, tu ayuda, consejo pero sobre todo sin tu saber escuchar. No solo has aportado conocimiento científico en este trabajo, si no que todo en esta tesis esta impregnado de tu animo, cariño, comprensión y amor. A ti Mai, eskerrik asko.

La tesis es algo tangible, consiste en plasmar los resultados de mucho trabajo en un papel para que perduren y otros puedan utilizar este trabajo y hacer avanzar el conocimiento. Sin embargo, yo prefiero quedarme con esos momentos efímeros; conversaciones, miradas y abrazos que durante estos años han acompañado al texto. Al igual que en el Pujani, ¿existe algo más eterno que una efímera tortisha de patatas?

Me dirijo a la ventanilla de billetes. Todavía no sé que tren tomaré…

RESUMEN

RESUMEN

La mielina es una estructura lipídica formada por los oligodendrocitos en el sistema nervioso central y que cumple dos funciones principales: por un lado permite la correcta transmisión del impulso nervioso y por el otro mantiene la homeostasis de los axones. En la esclerosis múltiple, enfermedad que afecta al sistema nervioso central, esta mielina se ve dañada por un ataque autoinmune. A modo muy resumido, linfocitos T activados cruzan la barrera hematoencefálica y atacan a la mielina provocando la muerte de los oligodendrocitos. Esto conlleva un fallo en la transmisión del impulso nervioso y un desequilibrio de la homeostasis axonal, lo que puede dar lugar a la degeneración del mismo. Sin embargo, en los primeros estadios de la enfermedad el organismo es capaz de regenerar la mielina; tras el daño desmielinizante se liberan una serie de señales que hacen que las células precursoras de oligodendrocitos proliferen, migren y se diferencien a oligodendrocitos, los cuales generarán nueva mielina alrededor de los axones. Mediante este proceso conocido como remielinización, se restablece el impulso nervioso saltatorio y se mantiene la homeostasis de los axones.

Desafortunadamente, con la evolución de la enfermedad este proceso termina por decaer. Aunque todavía no se conocen las causas exactas de la perdida del potencial remielinizante, estas parecen estar relacionadas con una población de células precursoras de oligodendrocitos escasa o con una pobre proliferación, migración o diferenciación celular. Actualmente, todos los fármacos disponibles para el tratamiento de la esclerosis múltiple se centran en prevenir el daño en la mielina. Estos son inmunomoduladores o inmunosupresores que evitan que el sistema inmune dañe la mielina, evitando de esta manera la desmielinización. Sin embargo, a pesar de que estos tratamientos son altamente efectivos, persiste una desmielinización subclínica. Además, con la evolución de la enfermedad, los pacientes terminan por entrar en una fase crónica en la que las lesiones desmielinizantes son abundantes. Por todo ello, se hace necesario el desarrollo de nuevos fármacos neuroprotectores o neurorreparradores, que combinados con los tratamientos inmunomoduladores o inmunosupresores disponibles, sean eficientes estrategias terapéuticas para los pacientes.

Actualmente existen dos líneas principales de investigación en relación con la promoción de la remielinización: el reemplazo de las células precursoras de oligodendrocitos o su estimulación para aumentar la proliferación, migración o diferenciación. La primera vía ha sido ampliamente investigada con resultados

prometedores. Sin embargo, el efecto positivo parece deberse más a una regulación inmune que a la propia regeneración de la mielina. Esto, unido a los riesgos de una terapia con células madre y al hecho de que la diferenciación celular parece ser un factor clave en el fallo de la remielinización ha hecho que la segunda vía sea postulada como un método más eficiente para estimular la regeneración de la mielina. Se han realizado distintos abordajes en este sentido, desde aproximaciones farmacológicas hasta el uso de vesículas extracelulares o de microRNAs.

Las vesículas extracelulares son partículas de entre 50 y 1000 nanometros liberadas por las células y que en función de su génesis pueden subdividirse en exosomas o micropartículas. Su principal función es la comunicación celular a distancia y para ello transmiten entre otros proteínas y material genético que producen cambios en la célula receptora. En cuanto a los microRNAs, son RNA pequeños de aproximadamente 22 pares de bases que tienen funciones reguladoras. Tanto las vesículas extracelulares como los microRNAs han sido relacionados con procesos de remielinización.

En el año 2014 el grupo liderado por Richard Kraig en la universidad de Chicago propuso que los exosomas derivados de sangre de ratas jóvenes eran capaces de fomentar la mielinización en modelos *ex vivo* y la remielinización en ratas adultas. Sin embargo, no demostraban si este tratamiento podría ser eficaz en la regeneración de la mielina en un modelo patológico. Por ello, nosotros quisimos testar el efecto de estos exosomas en el modelo animal de la esclerosis múltiple (el modelo de encefalitis autoinmune experimental). Con la misma aproximación metodológica, administramos por vía intranasal al modelo EAE dos concentraciones diferentes de exosomas aislados de sangre de animales jóvenes. Sin embargo no fuimos capaces de ver ninguna mejoría clínica en los animales tratados. Por ello decidimos analizar el potencial remielinizante de los exosomas en modelos más sencillos, como lo son los modelos *ex vivo* (Cultivo organotípico de cerebelo) o *in vitro* (Cultivos de células precursoras de oligodendrocitos (OPC; de sus siglas en inglés)). Nuevamente, los resultados indicaron que los exosomas no eran eficientes estimulando la remielinización en el cultivo organotípico (medida mediante microscopia confocal, western blot y resonancia magnética nuclear), ni en la diferenciación de las OPC (medida mediante reacción en cadena de la polimerasa cuantitativa (qPCR, de sus siglas en inglés) e inmunofluorescencia) . El efecto positivo de los exosomas derivados de sangre de animales jóvenes había sido relacionado con su

cargo, y más concretamente con la presencia de una serie de microRNAs, entre los que se encontraba el miR-219a-5p, que habían sido descritos previamente como inductores de la diferenciación de células precursoras de oligodendrocitos. Por ello, nos planteamos analizar el cargo de los exosomas mediante qPCR y micro arrays de expresión para ver si éstos contenían los microRNAs previamente descritos. Sorprendentemente, estos exosomas no solo no contenían el miR-219a-5p, sino que no contenían ningún otro microRNA relacionado con procesos de remielinización.

Este resultado nos permitió concluir que el ambiente podría estar relacionado con la carga de los exosomas, como estudios previos acreditaban y que su uso como posible terapia o vehículo necesita de una gran estandarización.

En este punto, decidimos confirmar si el miR-219a-5p por si solo era capaz de estimular la diferenciación de células precursoras de oligodendrocitos y por tanto la remielinización. Utilizando los mismos modelos *in vitro* y *ex vivo* que habíamos utilizado previamente, fuimos capaces de determinar que el microRNA por sí solo era capaz de estimular la diferenciación de los OPCs y por tanto promover la remielinización. Esto nos llevo a proponer que el factor clave en la capacidad remielinizante de los exosomas jóvenes podía deberse a la presencia en su cargo de miR-219a-5p.

En paralelo a la realización de este proyecto, pensamos que la monitorización de los animales del modelo animal podía ser mejorada por lo que diseñamos una nueva escala con la que evaluar a los animales. Además, generamos una aplicación móvil que permitiera la recopilación de datos de manera sencilla, evitando errores y facilitando su posterior análisis.

Retomando la línea anterior y tras los resultados obtenidos, decidimos determinar el papel que podía estar desempeñando miR-219a-5p en los pacientes de esclerosis múltiple. Para ello obtuvimos muestras de plasma de pacientes en dos estadíos de la enfermedad (brote y remisión) y analizamos los valores de miR-219a-5p mediante la técnica de la reacción en cadena de la polimerasa digital en gotas (ddPCR). La idea subyacente a este análisis consistía en que si el microRNA estaba involucrado en la regeneración de la mielina, este presentaría valores mayores en los estadíos de brote

puesto que el organismo se encuentra en un momento de remielinización para reparar las lesiones producidas durante el brote. A pesar de que no obtuvimos diferencias significativas entre los grupos estudiados, sí pudimos observar una tendencia a presentar valores mayores de miR-219a-5p en brote frente a remisión.

Dada la interesante información previa y con el fin de ir un paso más allá en el estudio de la capacidad remielinizante de miR-219a-5p decidimos generar el modelo animal de la enfermedad previamente utilizado en este trabajo y administrar el microRNA al animal. La administración directa del miR-219a-5p ya había sido testada en modelos de desmielinización toxica mediante vía intratecal, demostrando ser un eficiente factor pro-remielinizante. Sin embargo, nosotros quisimos administrar el microRNA de manera no invasiva, con el fin de que esta terapia fuera trasladable más fácilmente a la clínica, y por ello se realizó de la misma manera que se hizo con los exosomas derivados de sangre de animales jóvenes, por vía intranasal.

Consideramos que, dado que el microRNA tenía que llegar intacto hasta el sistema nervioso central, su encapsulación en sistemas de envío debía de ser primordial para protegerlo. Por ello, tras establecer una colaboración con dos centros tecnológicos locales (CICbiomaGUNE y TECNALIA) y una universidad extranjera (University of Oxford; donde realice mi estancia predoctoral), produjimos liposomas y nanopartículas cargadas con el miR-219a-5p como vehículos sintéticos y exosomas enriquecidos en miR-219a-5p como vehículo biológico. Tanto en los liposomas como en las nanopartículas el microRNA fue añadido a las formulaciones de la síntesis con el fin de que éste se encontrase embebido o en el interior de las vesículas. Por otro lado, los exosomas enriquecidos en miR-219a-5p fueron aislados de células HEK 293T transfectadas con el plásmido PlkO1 para que expresaran de manera constitutiva el microRNA. Cabe señalar que los exosomas contenían además del microRNA de interés, otras proteínas y material genético procedente de las células de origen.

Tras proceder a caracterizar morfológicamente los tres vehículos mediante técnicas de análisis de trazado de nanopartículas (nanoparticle tracking analysis) y de microscopia electrónica, analizamos mediante la técnica de la ddPCR la cantidad de microRNA-219a-5p que contenía cada vehículo, determinando que los liposomas tenían el mayor número de copias por partícula, seguidos de las nanopartículas y por último los

exosomas. En este punto, quisimos estudiar la capacidad de los vehículos de ser absorbidos por las células precursoras de oligodendrocitos. Para ello, tras generar el mismo cultivo primario de estas células que el mencionado en párrafos anteriores y tras marcar las partículas de manera fluorescente, fuimos capaces de determinar mediante inmunosfluorescencia que los liposomas eran captados más eficientemente que las nanopartículas y éstas a su vez que los exosomas. Sin embargo, los exosomas eran los más eficientes induciendo la diferenciación de las células precursoras de oligodendrocitos. Este dato fue muy llamativo puesto que los exosomas presentaban el menor numero de copias de microRNA por partícula y el menor porcentaje de captación. Sin embargo, como hemos dicho anteriormente y en comparación con los liposomas y las nanopartículas, los exosomas son vesículas biológicas cuya función es la de transmitir información de una célula a otra, por lo que se puede suponer que estos son mucho más eficientes que los vehículos sintéticos. Además, contienen otra serie de compuestos, como procesadores de microRNAs u otras moléculas que pueden aumentar la eficiencia del miR219a-5p.

Una vez seleccionados los exosomas como el vehículo más eficiente induciendo la diferenciación de las células precursoras de oligodendrocitos, decidimos administrarlos a los animales del modelo de esclerosis múltiple. Siguiendo el mismo procedimiento utilizado previamente, estos fueron administrados por vía intranasal. El estudio de la evolución clínica de los animales mostró diferencias significativas entre los animales tratados con los exosomas enriquecidos en miR-219a-5p y los tratados con los exosomas sin enriquecer. Además, tras analizar el patrón de inflamación de los animales pudimos determinar que la mejoría en la escala clínica no se debía a una regulación del sistema inmunitario de los animales, lo que hacia indicar que la mejoría se debía a un aumento en el nivel de remielinización, como así lo demostró posteriormente los estudios de resonancia magnética nuclear.

Estos resultados demostraban que el microRNA-219a-5p encapsulado en exosomas era capaz de inducir la diferenciación de las células precursoras de oligodendrocitos y que además estimulaba la remielinización en un modelo patológico de desmielinización. De esta manera pudimos concretar que los exosomas enriquecidos en miR-219a-5p podían ser una factible y posible estrategia terapéutica para pacientes de esclerosis múltiple. Asimismo, tras este trabajo, los exosomas se postulan como una vía efectiva de

administración de microRNAs al sistema nervioso central para otro tipo de patologías neurodegenerativas.

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ABBREVIATIONS

219-Ex	miR-219a-5p enriched exosomes
219-Lp	miR-219a-5p enriched liposomes
219-Np	miR-219a-5p enriched nanoparticles
AD	Axial diffusivity
BBB	Blood brain barrier
CNP	2´,3´cyclic nucleotide 3´ phosphodiesterase
CNS	Central nervous system
ddPCR	Droplet digital polymerase chain reaction
DiOC18	Dioctadecyloxacarbocyanine perchorate
DMEM	Dulbecco´s modified eagle´s medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco´s phosphate buffered saline
EAE	Experimental autoimmune encephalomyelitis
EB	Ethidium bromide
ESC	Embryonic stem cell
EV	Extracellular vesicle
Ex	Exosome
FA	Fractial anisotropy
HC	Healthy control
HEK293T	Human embryonic kidney cells 293T
HSC	Hematopoietic stem cell
iPSC	Induced pluripotent stem cell
lncRNA	Long non-coding RNA
Lp	Liposome
LPC	Lysophosphatidylcholine
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MD	Mean diffusivity
miRNA	microRNA
MOG	Myelin oligodendrocyte glycoprotein
MP	Microparticle
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis

MSC	Mesenchymal stem cell
MV	Microvesicle
ncRNA	Non-coding RNA
Ne-Ex	Non-enriched exosomes
Ne-Lp	Non-enriched liposome
Ne-Np	Non-enriched nanoparticle
NFL	Neurofilament
NG2	Neuron-glia antigen 2
NP	Nanoparticle
NSPC	Neural stem precursor cell
NTA	Nanoparticle tracking analysis
Ol	Oligodendrocyte
OLIG2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte precursor cell
PD	Parkinson disease
PDGFRα	Platelet derived growth factor alpha
PEG	Polyethylene Glycol
PGK1	Phosphoglycerate Kinase 1
PLGA	Poly(lactic-co-glycolic acid)
PLP	Proteolipid protein
PPP	Platelet poor plasma
PVA	Poly vinyl alcohol
qPCR	Quantitative polymerase chain reaction
RA	Radial diffusivity
RNA	Ribonucleic acid
RPL13α	Ribosomal protein L13 α
RRMS	Relapsing-Remitting multiple sclerosis
RVG	Rabies virus glycoprotein
siRNA	Small interfering RNA
snoRNA	Small nuclear RNA
soRNA	Small nucleolar RNA
WB	Western blot
WT	Wild type

NOTE: During this work microRNA 219 is mentioned several times. However, we want to point out that depending on the reference source, the nomenclature changes and miR-219 or miR-219a-5p are used as synonyms. Nevertheless and according to miRBase, miR-219 was previously used to refer to miR-219a-5p. We did not want to change the nomenclature used in the original paper and both can be found during the text referring to miR-219a-5p.

INTRODUCTION

Humans are complex organisms formed by more than 30 trillion cells each. These cells form structures named tissues, which reorganization is responsible of the appearance of organs. These organs form systems such as the immune system, the cardiovascular system or the excretory system, among others. This complex machinery allows us to be born, grow, reproduce and die, but also to interact to each other in the most complex society ever discovered in the animal world.

The nervous system is responsible for the correct running of all these parts, interacting with all of them and making unconscious decisions for that. In addition, our brain can not only receive and interpret inner information, but also external information taking conscious decisions. As an example, when we are having a conversation with a colleague, we are breathing, our heart is pumping blood and our stomach is digesting the delicious food we had one hour before our meeting. Moreover, the brain is processing the information that our colleague is presenting us about the last experiment and we are trying to analyse these results. Our hand is also moving extremely fast to write down the most important conclusion of the work. In addition, we are able to watch the slides that are shown on the screen. In summary our brain is working hard.

But, how can the brain make so many complex things at the same time? A network of neurons is sending information from one to another activating and inhibiting concrete cells of this network in order to be able to conduct all the required actions. The transmission of the information by the neuron is called nerve impulse.

1. The nerve impulse transmission

Neurons are cells formed by a soma, in which the information is received and processed, and an axon that transmits the information by an electric potential change that activates the following neuron. This is called excitatory potential and consists of the modification of the ionic concentration that produces an imbalance in the charge of both intra and extracellular spaces. Basically, sodium and potassium pumps, which are located in the membrane of the axons, are responsible for it. On the one hand, potassium concentration is higher in the intracellular space. Interestingly, axons´ membranes are selectively permeable to potassium ions, allowing potassium to scape from the cell to compensate its excess. This creates a positive charge outside the cell and a negative

charge inside the axon (Figure 1; 0). The difference on the charge is called action potential. On the other hand, sodium concentration is higher in the extracellular space. A concrete stimulation of the cell allows sodium membrane pumps to pump sodium into the cell, as a mechanism to compensate the negative charge (Figure 1; 1). This inverts the charge generating a positive charge inside the cell and a negative one outside. This is called depolarization of the membrane and lasts around 1 millisecond (Figure 1; 2). This process activates adjacent sodium pumps spreading the nerve impulse along the axon and stimulating the following cell. Again, potassium pumps try to compensate the charge by pumping potassium to the extracellular space and restoring the action potential in a process called repolarization (Figure 1; 3). However, there is an intermediate step which creates a hyperpolarization of the axons due to an excess of potassium that flow outside the cell (Figure 1; 4). Then sodium-potassium pumps restore the initial ionic distribution in the intra- and extracellular space (Figure 1; 0). The whole process takes place in around 4 milliseconds [1].

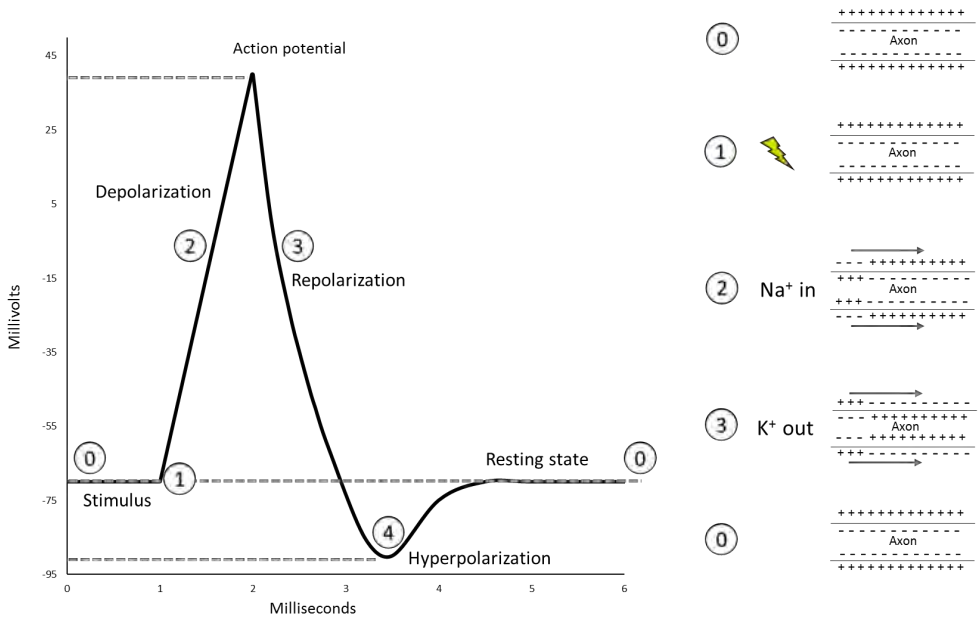

Figure 1: Schematic representation of the action potential and the changes in the axon polarization that take place during it.

However, when the action potential travels along the axon, ions leave the axon and its magnitude decreases proportionally to the travelled distance [2]. To prevent this, most axons in the central nervous system (CNS) are covered by myelin, which is an oligodendrocyte (Ols) membrane extension that wraps axons. Myelin is a potent

insulatior which decreases capacitance and increases electrical resistance along the axon, preventing on the one hand the electric potential from leaving the cell [3], and allowing on the other hand the action potential to travel without decreasing the speed of transmission. Additionally, myelin is disrupted every millimetre in a structure called node of Ranvier in which axons are naked and where action potentials are generated. Interestingly, nodes of Ranvier are enriched in sodium and potassium channels, facilitating this task [4]. In this way, action potentials "jump" from one node to another, in a process called saltatory nerve impulse transmission, allowing a transmission speed of 120 meter per second (much faster than the 10 meters per second of non-myelinated fibres) [2] (Figure 2). In addition to this, myelin protects and gives trophic support to neurons. More concretely, oligodendrocytes have been proposed to support axons with energetic metabolites such as glucose or lactate [5,6]. As an example, glycolytic oligodendrocytes have shown to maintain long-term axonal integrity [7].

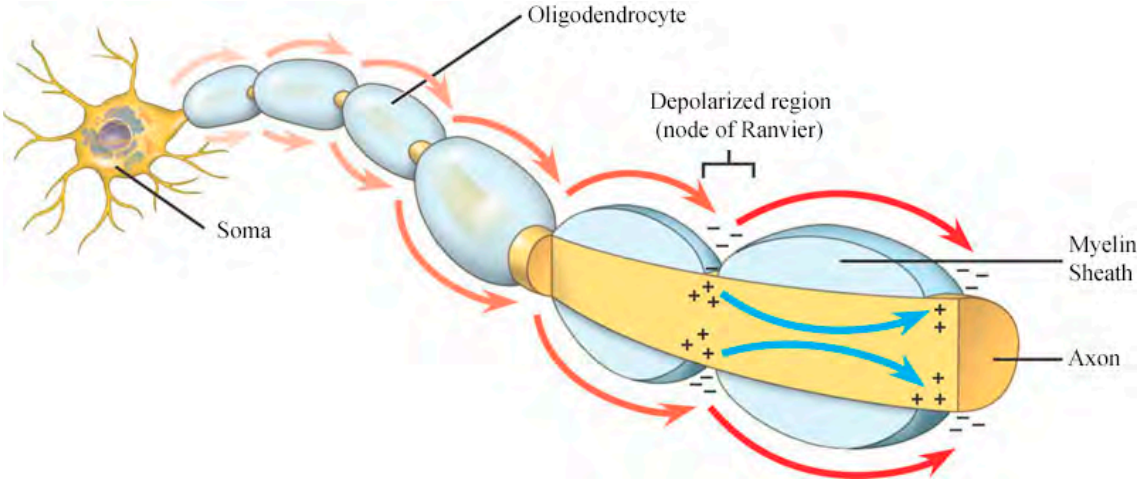

Figure 2: In this image, the soma and the myelinated axon of a neuron are shown. Red arrows represent the saltatory nerve impulse transmission. Adapted from Antrik.org [8]

In summary, myelin provides metabolic support to the axon and allows the transmission of the nerve impulses fast and with low energetic requirements. To understand these functions and as it can be appreciated in Figure 3, myelinating oligodendrocytes generate a growth zone that attaches and encircles an axon segment generating a multilayered and spiral structure [9]. One oligodendrocyte can myelinate more than one axon, and one axon can be myelinated by more than one oligodendrocyte. Myelin is mainly formed by cholesterol, lipids and proteins including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) that play a role in myelin compaction [10].

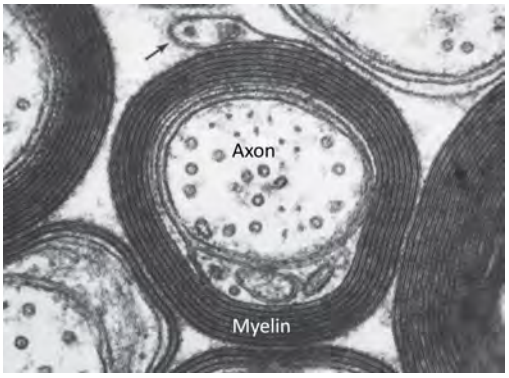

Figure 3: In the centre of the image and axon and the myelin can be appreciated. Adapted from MacKay, 2016 [11].

2. Myelin and Multiple Sclerosis

Multiple sclerosis (MS) is a central nervous system disease in which myelin is damaged by an autoimmune attack. It is not clear which factors are implicated in the development of MS, but a combination of genetic predisposition [12] and environmental factors (such as vitamin D, time of birth or smoking [13–15]) which lead to an immune dysregulation, have been described to be behind MS [16]. It seems to be clear that activated T cells cross the blood brain barrier (BBB), and react against the myelin. The BBB is an anatomical barrier that separates the blood from the glial cells and that carries out several important functions such as the regulation and protection of CNS cells. However, as it is mentioned in [17], the BBB is more a concept rather than a proper barrier, with dynamic properties that allows for instance immunological surveillance. Lymphocytes have been described to be able to cross the BBB under normal conditions by several routes [18]. However, an increased migration of autoreactive lymphocytes across the BBB together with an aberrant immune response are thought to be the causes of multiple sclerosis outbreak and therefore myelin damage [19]. This damage causes demyelinating lesions, also known as plaques. While they are distributed heterogeneously along the CNS; they appear mainly in the white matter, which are the areas with more myelin content.

Demyelinating lesions are responsible, at the very beginning, for producing an inadequate nerve impulse transmission that can concur with the first clinical symptoms. Although clinical manifestations might vary from one patient to another, we could say that these are related with sensitive and motor alterations, such as paresthesia, hypoesthesia or optic neuritis [20]. In addition to this, naked axons are exposed to

potential harmful factors and are deprived from trophic support, which can lead to axonal death.

Fortunately, at the first stages of the disease, the body is able to regenerate the myelin in a complex process called **remyelination**. It is of extreme importance because myelin regeneration will restore both metabolic support to the axon, avoiding its degeneration, and nodes of Ranvier that are required for a proper nerve conduction and function [21]. This is, remyelination restores nerve impulses conduction [22].

Demyelination induces the secretion of signalling molecules and cytokines that activate astrocytes which in turn secrete a range of factors that activate progenitor cells and induce their proliferation [21,23]. This activation is related to an increase in transcription factors that finally induces the regenerative process [24,25]. These progenitor cells are adult oligodendrocyte precursor cells (OPC) which are abundant throughout the CNS (being a 6% of the total number of the cells in this region of the body [26]) and that have been described as the major source of new oligodendrocytes [27]. In addition, OPC have been described as self-renewing multipotent cells, being proposed as adult CNS stem cells [28], highlighting the role that they can play after a demyelinating insult.

Activated OPC migrate and colonize areas of demyelination in order to differentiate and generate the number of required oligodendrocytes to regenerate myelin [21] (Figure 4). Microglia, monocyte-derived macrophages, astrocytes and OPC themselves release regulators of migration and mitogens that induce the process [21]. In addition, the clearance of myelin debris after demyelination made by macrophages is of extreme importance for OPC differentiation as myelin debris inhibits this process [29–31]. Finally, differentiation phase begins when OPC exit the cell cycle [32] and oligodendrocytes generate new compact myelin that wraps axons.

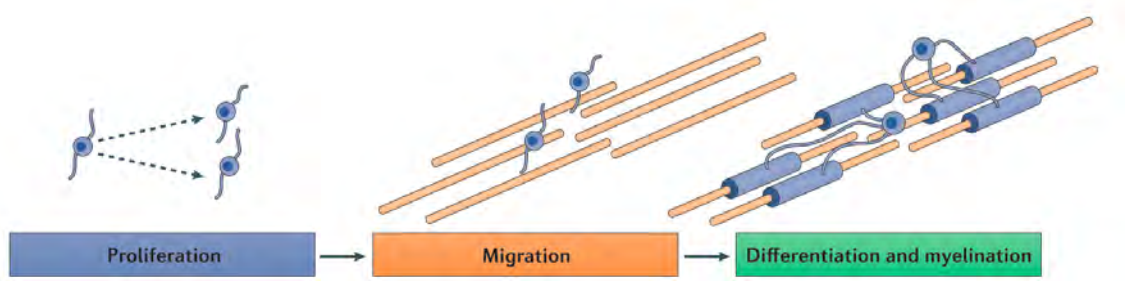

Figure 4: Schematic meyliantion process: Proliferation of OPC is follow by their migration into the lesions, their differentiation and finally the generation of new myelin. Modified from Stangel M. et al 2017 [33].

However, as it has been described, myelin generated in remyelination is thinner and with shorter internodal distances than myelin generated during development, reducing its effectiveness [34,35] (Figure 5). This can be expressed by the g-ratio, a number that relates the axon diameter to the myelin thickness. These differences between both processes have been associated with dynamic growth, having in mind that during development axons and myelin are growing at the same time sending information from one to another. On the contrary, in remyelination axons are perfectly formed and no variation in their size can be produced [36].

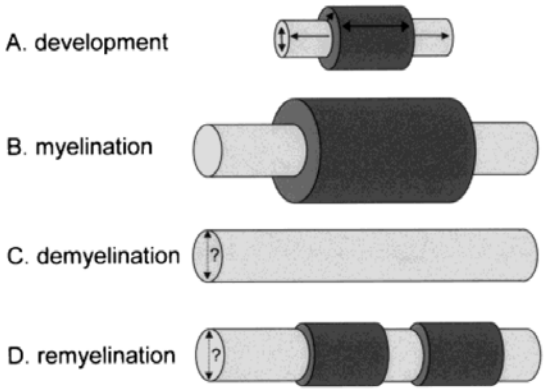

Figure 5 Differences shown in myelin during development (A), in an adult axon (B), after demyelination (in which no myelin is present)(C) and after remyelination (D), when myelin is thinner and with shorter internodal distances. From Franklin and Hinks 1999 [36].

Remyelination has not been described to be uniform along the CNS as subcortical lesions tend to remyelinate better than perivascular ones [37]. In addition, it can vary from one patient to another [38]. Moreover, remyelination has been found to loss efficiency with disease evolution as 80.7% of early lesions are remyelinated compared to a 60% of chronic ones [39].

Related with this last point, after several cycles of demyelination-remyelination, and together with disease progression and aging, remyelination process terms to fail [40]. It is not exactly clear why remyelination fails but researchers have postulated that it is related with a decrease in the numbers of OPCs, with an inefficient migration, with an inexistent or poor differentiation or with a combination of all these factors [41]. However, other evidences indicate that there is no an aging-related decline in OPC but a lower recruitment and differentiation levels of these cells [42]. Remarkably, more evidences point out that differentiation might be the key factor, as the promotion of OPC recruitment in experimental demyelination mice do not improve remyelination [43]. In addition, chronic no remyelinated lesions have been described to contain OPC but not oligodendrocytes [44–47], highlighting the role that OPC differentiation stimulation can play in remyelination therapies.

As it has been previously introduced, remyelination failure makes naked axons to be unprotected, which at final term cause neuronal death and therefore neurodegeneration. This is an irreversible process that when occurs, neuronal circuits get interrupted (Figure 6).

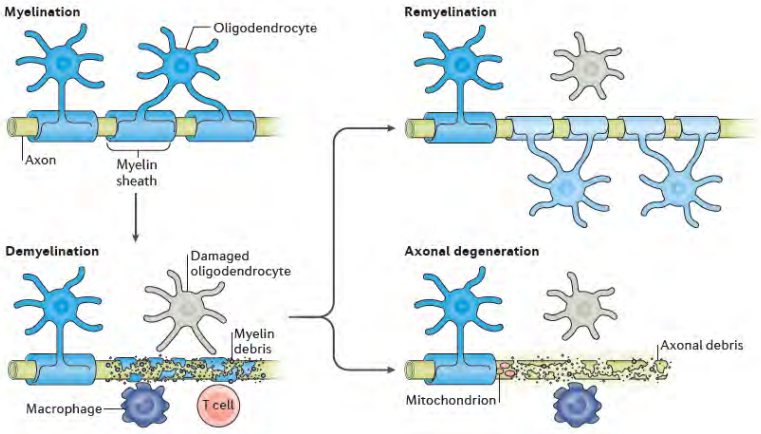

Figure 6: Following demyelination, axon remains intact enabling the regenerative response of remyelination. However, in the absence of remyelination, saltatory conduction can not be restored leading to axonal degeneration. From Franklin 2017 [21].

MS is an heterogeneous disease and the evolution that has been explained above with cycles of demyelination-remyelination with progressive disability of the patient is called relapsing-remitting MS (RRMS) and 85% of the patients develop this form of disease. It is usually followed by a secondary progressive form in which neurodegeneration is the main characteristic. Interestingly, therapies for RRMS are focused in protecting myelin to be damaged by developing immunomodulatory or immunosuppressive treatments that kill, attenuate or disable overactive and autoreactive lymphocytes [48]. Although they are very effective in this task, a subclinical neurodegeneration progresses and age-associated decline in remyelination efficiency finally increases patients´ disability. Unfortunately, progressive MS is nowadays untreatable, and a big effort is being done trying to discover new and promising treatments to improve remyelination and prevent neurodegeneration.

3. Models for studying myelination demyelination and remyelination

Adapted from Osorio-Querejeta et al 2017 [49] .

Experimental models are needed in order to understand in deep myelin formation, demyelination and remyelination processes, with the final goal of developing therapies that can promote remyelination and prevent axonal degeneration. We should bear in

mind that no model, no matter how sophisticated, can mimic all physiopathological processes that occur in humans. Nevertheless, there are models that simulate several aspects of myelin-related diseases and enable us to study myelination, demyelination and remyelination as well as the potential of remyelinating therapies. Different models can be used depending on the final goal of the research.

In this section, several models will be presented, in order of complexity, emphasizing the main characteristics and potentials of the most commonly used models for studying the different pieces of the myelin puzzle.

3.1. In vitro models

3.1.1. OPC culture

The isolation and culture of OPCs can be a helpful tool for understanding the mechanisms involved in the development of myelin and studying the effect of new therapies on these types of cells in relation with migration, differentiation, survival and proliferation [50,51].

A wide variety of protocols have been elaborated for obtaining these cells from human tissue, namely, cryopreserved umbilical cord [52], foetal brain [53] brain biopsies and embryonic stem cells (ESC) [54]. These sources enable to work with human OPCs but they have the drawback that samples are difficult to obtain and their use raises ethical concerns. Due to this inconvenience, induced Pluripotent Stem Cells (iPSC) has emerged as an alternative for obtaining human OPCs [55] (and also rodent OPCs [56]). Working with iPSC allows working with human derived OPCs and with a large number of cells. Moreover, iPSC have their goal in therapeutics since they can be obtained from the own patient allowing autologous transplantation. Another possibility is to obtain OPCs from the optic nerve [57] or brain cortex of young and adult rodents.

There are a variety of methods for isolating OPCs: Oligodendrocyte Selection Kit [58], Magnetic-activated cell sorting [51], Manual method [59], Immunopanning [60], Fluorescence activated cell sorting [50] and Immortalized cell line [61]. All these methods can achieve a good quality of OPC and high content cultures. Therefore, the choice of the method

should be accomplished based on the equipment and resources of each lab. Once the OPC are isolated, differentiation, proliferation, survival or migration assays can be performed. To characterise the ability of a drug to promote the migration of the cells, after culturing them in transwells, the number of cells that did cross the membrane can be measured.

In order to characterise each stage of OL differentiation after the administration of a treatment, various types of markers can be used for specific proteins, including platelet derived growth factor (PDGFR) α, neuron-glial antigen 2 (NG2) and A2B5 for OPCs, 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP), O1 and O4 for pre-OLs; myelin basic protein (MBP), Proteolipid Protein (PLP), Myelin Oligodendrocyte glycoprotein (MOG) and Myelin-associated glycoprotein (MAG) for OLs; and oligodendrocyte lineage transcription factor 2 (OLIG2) for all OL-lineage cells, among others (Figure 8). For survival or proliferation a specific markers of cell death (caspase-3) or cell proliferation (5-bromodeoxyuridine) can be used respectively. Then, results can be analysed by fluorescence microscopy. Alternatively, flow cytometry can be used, facilitating multiparametric analysis, however, it does not enable the visualization of the cell morphology. Gene expression analysis can also be used to characterize the culture (*Figure 7*) [62,63].

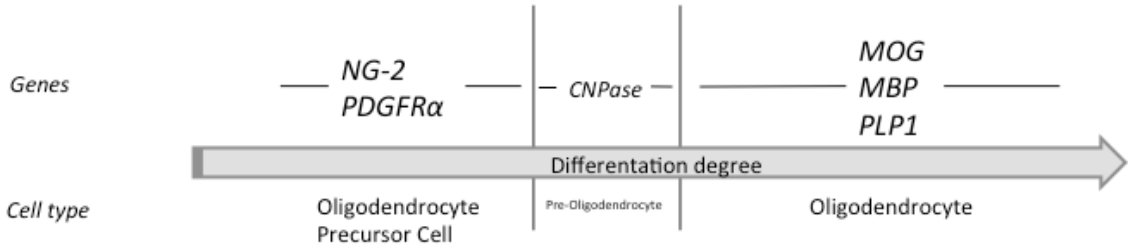

Figure 7: OPC differentiation stages at gene expression level. Modified from Osorio-Querejeta et al. Submitted.

This *in vitro* model based on OPC culture is simple, low cost and high-throughput. They represent a first step towards ascertaining whether treatments have positive effects on the cells responsible for myelination and remyelination and understanding the mechanisms involved. Moreover, since the cells are not influenced by other types of cell or the environment, exposure of these cells to a drug simplifies the interpretation of

results [61]. With this approach, we can lay the foundations for further work with more complex models.

3.1.2. "Axon-based models"- Co-cultures

These models of myelination involve obtaining axons, either natural or artificial, in which to test the myelination ability of cells under study, as a potential therapy for demyelinating diseases. It is possible to administer a drug together with the cells as a treatment, and then study their influence in terms of myelin production by these cells. Below, we describe two methods for obtaining axons: from spinal cord explants and by producing synthetic axons.

3.1.2.1. Spinal cord explants

In this model spinal cord explants (mainly from rat or mouse embryos) or dorsal root ganglion explants [64] are cultured allowing neurons to extend their axons outwards, serving this as a substrate for myelination by exogenous cells added to the culture [65,66]. Using these models, it is possible to assess the capacity of myelinating cells to form myelin around axons [67], as well as the effect of drugs on these cells under neuro-glia interaction.

Once the explants are cultured [68], myelinating cells are added. The effect of these cells can be analysed by measuring the amount of myelin by using immunofluorescence microscopy. In parallel the myelin g-ratio can be determined by electron microscopy [6769].

This is a myelination model that allows researchers to study interactions between neurons and myelinating cells during the myelination process. However, we need to bear in mind that in these explants, besides neurons, there are other types of cells that can also influence the myelination process [70]. For this reason, some authors classify these as *ex vivo* models.

3.1.2.2. Synthetic axons

In order to avoid interactions between myelinating cells and other cells from explants a variant of the previous model type was developed involving inert or synthetic axons[71].

Synthetic axons can be obtained by fixing axons with paraformaraldehyde[72] or by producing artificial nanofibers that mimic axons. Artificial nanofibers have been developed with a range of techniques, going from glass microfibers coated with a glial cell matrix [73] continuing with vicryl microfibers [74], finishing with polystyrene [71,75] or polycaprolactone [76] nanofibers designed using electrospinning. A variant of this model uses micropillar arrays, formed in a 96-well plate, which contain 50-µm-diameter conical structures that cells are able to myelinate [77].

These artificial fibres can be coated with a selected substance (from proteins to microRNAs) [78], in order to study in depth their effect in myelination [75], facilitating the subsequent design of remyelinating therapies based on these factors.

3.2. *Ex vivo* models

3.2.1. Organotypic cultures

These types of cultures involve the growing of tissue in three dimensions mimicking the structure and cell types of living organs, making them an intermediate step between cell cultures and animal models. These cultures enable the development of models for certain neurological diseases such as ischaemia, Parkinson´s disease and Huntington's disease, as well as MS [79].

Several CNS structures including brain, cerebellum and spinal cord can serve as the source for developing myelination, demyelination and remyelination cultures, each with their own characteristics [80]. The cerebellum provides homogeneity in the type of axons (mostly Purkinje cell axons), while the brain and the spinal cord have more axonal variety in terms of type and diameter. In particular, the spinal cord may be a choice for OPC migration studies [81].

With the help of a vibratome or a tissue chopper (McIlwain), approximately 300-µm slices of tissue are obtained and then cultured on membranes (specifically designed for this type of culture) for subsequent demyelination. The first report of induced demyelination dates from 1959, in which Bornstein *et al.* describe that they succeeded in demyelinating a cerebellar organotypic culture by adding serum derived from animals with Experimental Autoimmune Encephalomyelitis [82] an animal model of MS.

Nowadays lysophosphatidylcholine, a detergent that mainly destroys myelin, is used as a demyelinating agent [83]. After removing the detergent, the OPCs present in the culture are capable of regenerating myelin enabling remyelination studies [81].

This type of model allows assessing the regenerative capacity and speed of endogenous cells to regenerate myelin under the influence of different drugs. It is also possible to analyse remyelination by cell therapy. For this, cytosine arabinose should be added to the culture to supress the proliferation of endogenous cells [84] and avoiding therefore the remyelinating potential of endogenous OPCs.

Culture growth can be followed with time-lapse imaging [81,85]. At the end of the culture period, the tissue is fixed and immunofluorescence staining is performed to label axons and myelin. By confocal microscopy the area occupied by myelin-coated axons compared to the total area of axons is analysed obtaining semi-quantitative data [70] (Figure 8). Internodal length and g-ratios analysed by electron microscopy can be indicators of the remyelination quality.

To achieve a favourable culture growth, young animals should be used. Nevertheless, it should be also taken into account that demyelinating diseases do not tend to occur in early stages of development. Finally, although the structure of the original tissue is maintained, these models do not replace *in vivo* models. However, they are useful tools for screening treatments in advance of *in vivo* testing, thereby reducing the number of animals to be used [79].

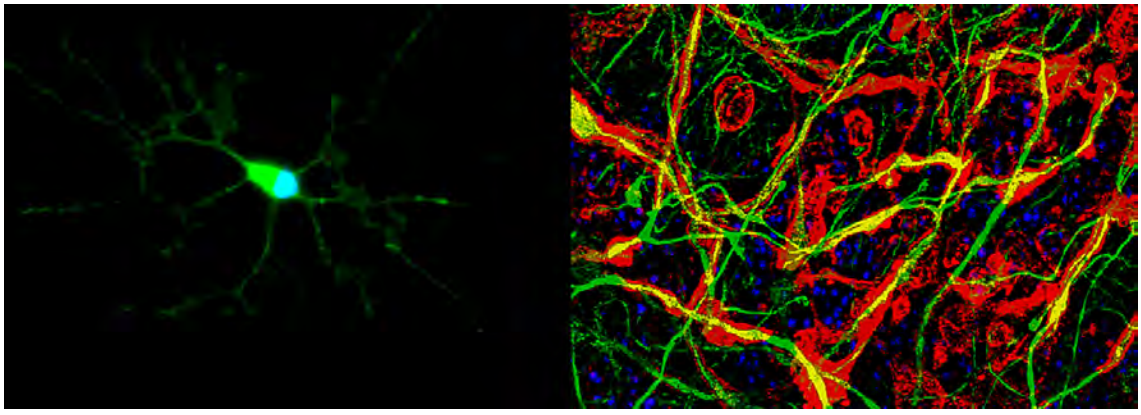

Figure 8: Left: Oligodendrocyte precursor cell image labelled for NG-2 (Green) and Hoechts (blue). Right: Cerebellar organotypic culture confocal image stained for MBP (red), Neurofilament (green) and Hoechts (blue). Yellow represents the colocaliation of

myelin and axons. Notice that both images have been modified to obtain more intense colours.

3.3. In vivo models

3.3.1. Zebrafish

Zebrafish is a vertebrate animal that has been widely used in developmental studies. Given the fact that it gets to the adult stage quickly and that is transparent, it makes possible to visualise internal structures noninvasively. In addition, myelination in this fish species is similar to the one that takes place in mammals [86]. In fact, it has homologous genes involved in the myelination process [87]. Due to these reasons, this animal has been used in myelination studies and in drug screening [88].

Further, transgenic zebrafish have been developed in which OLs express membrane-targeted green fluorescent protein [89], and in which both OLs and Schwann cells express a fluorescent protein making them visible[90]. These models are useful for studying myelination *in vivo* [90], but do not allow analysis of remyelination due to the lack of physiological demyelination. To perform remyelination studies in this model, demyelination has been achieved by destroying myelin with laser microsurgery [89], or by producing transgenic individuals such as the system reported in 2013 by Park's research group, in which OLs die after exposure to metronidazole [91]. After the microsurgery or when metronidazole is taken off, remyelination process starts.

These processes can be monitored *in vivo* by time-lapse confocal microscopy without the need of sacrificing the animal, provided that OLs express a fluorescent protein [89]. In addition, post-mortem immunofluorescence can be performed to search for the proteins of interest.

This model provides a rapid method for assessing candidate agents for the treatment of demyelinating diseases, given that it is possible to monitor OLs *in vivo* [87,92]. Related with this, Franklin's research group developed a screening platform based on this type of model. They identified the most promising compounds to be tested later in mammalian models [92]. However, these models are not suitable for testing cell therapy, given the technical difficulties involved.

3.3.2. Mammalian animal models

A great variety of models allow us to study remyelination in mammals. The main advantage of using mammals is their closeness to humans. Here, we will focus on murine models, which are the most widely used ones. These have been carried out for studying pathological processes associated with demyelinating diseases and remyelination, and can be classified into three types: models of toxicity, models of viral infection and immune response based models.

The ways of obtaining data are similar for all the murine models listed below, with some exceptions that will be specified later. During the development of the model, magnetic resonance imaging can be used for monitoring animals. After sacrificing animals, histopathological studies can be performed to detect myelin by using Luxol Fast Blue, immunohistochemical or immunofluorescence analyses. The remyelination quality can be studied determining the g-ratio of axons by electron microscopy. Another possibility is to extract the CNS, do an emulsion with the tissue and analyse the cell content by flow cytometry or gene expression [62]

3.3.2.1. Models of toxicity

Toxin-induced local lesions: These are models of localised acute cytotoxicity, in which OLs are depleted[93] following an stereotaxic injection of lysophosphatidylcholine (LPC) or ethidium bromide (EB), among other agents. EB is an intercalant dye that produces the loss of OLs and astrocytes, but does not affect axons [94]. LPC alters the membrane composition, specifically of OLs, due to its membrane fusogenic properties, destroying the myelin [95,96]. As a consequence, microglia and macrophages move towards the generated lesions, a reactive astrogliosis in generated, axonal homeostasis is disturbed and OPCs proliferate and migrate to the damaged tissue [97]. There is no consensus regarding the presence of concomitant demyelination and excessive inflammation [94,98]. Further, lesions can be directed to different regions of the CNS and peripheral nervous system, including the dentate gyrus [99], sciatic nerve [100], spinal cord [97] and centrum semiovale [101].

These toxin-induced models are highly reproducible [98] and useful for studying the effect of different drugs on remyelination alone, in the absence of inflammation. However, it needs to be taken into account that there is necrotic damage around the injection site [98,101].

Models of general toxicity: These models are based on the production of demyelinating lesions by neurotoxic, chemical, or biological agents administered through the diet. The most widely used toxin is cuprizone, a copper-chelating agent that induces OLs apoptosis, and as a consequence, demyelination. Though the mechanism involved remains unclear, it has been proposed that it produces errors in the mitochondrial respiratory chain [102]. In contrast to Experimental Autoimmune Encephalomyelitis, that will be explained further, T cells do not play a role in the generation of this model, and not all the regions of the brain are affected in the same way [103]. Depending on the duration of cuprizone treatment, 6 or 12 weeks of treatment, it is possible to produce an acute or a chronic model, respectively. In both of them, after stopping the cuprizone treatment, there is a spontaneous remyelination of the lesion, this being slower and more limited in the chronic model [102,104]. A variation of these models has been developed, using curpizone in combination with rapamycin, achieving a complete demyelination and slower remyelination when compared to cuprizone alone [105].

The model is simple and reproducible, but age, sex and the species of animals used, can modify the results [102]. For instance, remyelination is slower and more limited in old animals[103].

The model enables to study demyelination and remyelination processes that occur in the CNS and the effect of drugs and cell therapies [104]. However, it should take into account that to study cell therapies it is necessary to administer cells after withdrawing cuprizone from the diet in order to avoid these cells to be damaged. Further, we should emphasize that precursor cells are not affected by cuprizone and hence they will compete with the administered cells to repair the lesions [102].

3.3.2.2. Models based on viral infection

These models are based on the hypothesis that some viral infections may cause demyelinating diseases such as MS. The most commonly used viruses are the Semliki Forest virus [106] and Theiler's murine encephalomyelitis virus [107]. Semliki Forest virus was first isolated in mosquitoes and rarely affects humans. This virus can infect OLs and due to the highly virulent feature of the M9 mutant, the avirulent A7 strain is more frequently used. After the virus has infected oligodendrocytes, the immune system responds by attacking OLs, generating in this way demyelination. Theiler's murine encephalomyelitis virus leads to neurological pathological features (such as paralysis and encephalomyelitis) in a wide range of animal species. The most commonly used strains include the virulent GDVII and the less virulent ones, Daniels and BeAn strains.

Models of viral infection allow us to explore the potential effect of immunomodulatory and remyelination therapies on CNS cells infected by a neurotrophic virus [108]. This feature is interesting since some authors described that MS may be cause by a viral infection[109]. However, these models are not extensively used, due to the long incubation periods between infection and the onset of the symptoms, the high mortality rates among infected animals, and the technical difficulties related with the manipulation of viruses. Lastly, these models are not appropriate for testing cell therapies, as the virus would have tropism for injected cells, being affected in the same way as endogenous cells.

3.3.2.3. Models based on immune response: Experimental Autoimmune Encephalomyelitis (EAE)

The first reference to this experimental model dates from 1933, when Berry’s research group succeeded in causing inflammation and demyelinating lesions in the CNS of monkeys after several consecutive intramuscular injections of brain emulsion [110]. The mechanism involved in the generation of the models were not clearly elucidated, but now it is known that this condition is mediated by specific T cells against myelin antigens, and that it has clinical and histopathological similarities to MS [111]. In fact, this is the experimental model that exhibits most similarities to the human disease and the most commonly used for studying remyelinating drugs.

The features it shares with MS include the destruction of myelin, the development of lesions over time and across the CNS, though mainly perivascular in scattered foci, and the presence of immunoglobulins in both the cerebrospinal fluid and the CNS. Although, demyelination and remyelination in these models are less extensive and more acute than in humans [112], this model is considered the gold standard for the study of MS. It is used for preclinical proof of concept of new pathways and mechanisms of action in the pathogenesis of autoimmune diseases. Finally, many of the findings with this model have been applied to treatments in patients.

There are two subtypes of this model. The active one, in which animals are immunised against a myelin peptide, together with Freund´s adjuvant and pertussis toxin. Depending on the peptide and the animal host and strain, there are a range of different models, and these are widely reviewed in the literature [80,108,113]. The passive or the adoptive cell transfer model one is produced by injecting specific active lymphocytes against myelin, which are obtained from the lymph nodes of animals that have undergone active immunisation.

We have to be cautious when analysing the results obtained with this model, since there are many cases of therapies that have been proven to be effective in the animal model but did not shown the same positive effects in humans [114]. Moreover, this type of model fails to predict adverse effects of treatments, especially long term effects, due to the short periods of time of the model [79,115]. On top of that, it is an expensive animal model in terms of numbers of animals that need to be used, time and money [81]. Its limitations are mainly related with the lack of understanding of all the mechanisms involved in the pathogenesis of the model, and the short time frame of the model for studying chronic conditions. Finally, this is the most complex model of all those mentioned in this section and this sometimes makes the interpretation of findings difficult [116].

Remyelination failure is more characteristic of the chronic forms of MS. For this reason, chronic EAE models are more suitable for studying remyelination due to the more similarities with the secondary progressive MS[114]. These models are the ones produced by MOG peptide in Dark Agouti rat and C57BL/6 mouse [117,118].

The principal information obtained from this model is the animal clinical score. This information is subjected to the observer's bias; therefore it is essential to carry out double-blinded experiments as well as appropriate statistical analyses [119].

3.3.2.4. Other models

In 2015, Gocke's research group published a study that combined the EAE model with a toxin-induced demyelination model. In this new model, demyelination occurs in the absence of neurodegeneration which, according to the authors, makes possible to assess remyelinating therapies that have previously been difficult to study given the neurodegeneration in EAE [120].

Lastly, there are other models that are based on the use of genetically modified animals, in which T cells express specific receptors of myelinating cells, in order to produce demyelinating lesions. However, these models are not yet commercially available, rarely used, and require long periods of development.

3.4. Choosing the appropriate model

Models to study myelination, demyelination and/or remyelination have been recapitulated. In order to obtain valuable results and proper conclusions, it is of high relevance to choose an appropriate model. For that, the following points whould be taken into account.

- *Myelination, demyelination and remyelination:* Although the final goal is to induce the production of new myelin after damage, not all the models allow us to analyse this directly. Nevertheless, all the models do provide information needed to improve our understanding of the mechanisms related to remyelination processes.
- *What question do we want to answer?* Not all the models can provide answers to all questions. For this reason, it is important to clearly define which data we would like to obtain and chose the most appropriate model for this aim (Table 1).
- *Models are complementary:* It is advisable to use more than one model, since different models can provide complementary information that increases the quality of the results. Each model has strengths and weaknesses, and provides

different types of data, which well used allows the researchers to obtain more robust conclusions.

- *The most complex is not always the best*: The greater the complexity of the model, the more appropriate for studying remyelination. However, understanding the mechanisms prior to remyelination with simpler models may help identifying and improving our comprehension of processes that lead to the development of remyelinating therapies.
- *From simple to complex models:* The simplest models allow testing higher numbers of molecules/therapies, since it is easier to obtain larger sample sizes and there are few ethical constraints. Moreover, those models are easier to manage and less expensive. Then, the agents that have yield positive results can be analysed in more complex models, which mimic better the mechanisms occurring in humans. In this way, as we move to more complex models we reduce the number of candidate agents that eventually are going to be tested in clinical trials (Figure 9).
- *We cure mice; what about humans?* Treatments studied in experimental models with positive results not always show the same effects in patients, being not effective or producing serious side effects. It is necessary to remember that we are working with *in vitro, ex vivo* and/or *in vivo* experimental models and that it is indispensable to be careful extrapolating the results to humans.

	in vitro models			*ex vivo* model	*in vivo* models				
	OPCs	Spinal cord explants - OPCs	Synthetic axons	Organotypic cultures	Zebrafish	Local toxicity	General toxicity	Viral infection	EAE
Evaluation of large numbers of agents	+++	++	++	+ +	+	+	+	+	+
Short periods of development	+++	+++	+++	+++	++	++	+	+	+
Presence of inflammation	0	0	0	0	+	+	+	+++	+++
Drug screening	+++	++	++	+++	+++	+++	+++	+++	+++
Cell therapies screening	0	++	+++	++	+	++	+	+	+++
Ethical constraints	++	++	++	++	+++	+++	+++	+++	+++
Demyelination studies	0	0	0	++	++	+	+	+	+++
Remyelination studies	0	0	0	+++	++	++	+++	+++	+++
Myelination studies	+++	+++	+++	+	+++	0	0	0	0
Complexity	+	++	++	++	+++	+++	+++	+++	+++
Closeness to humans	0	0	0	0	+	++	++	+++	+++

Table 1: Summary of the main characteristics of models for studying myelination, demyelination and remyelination

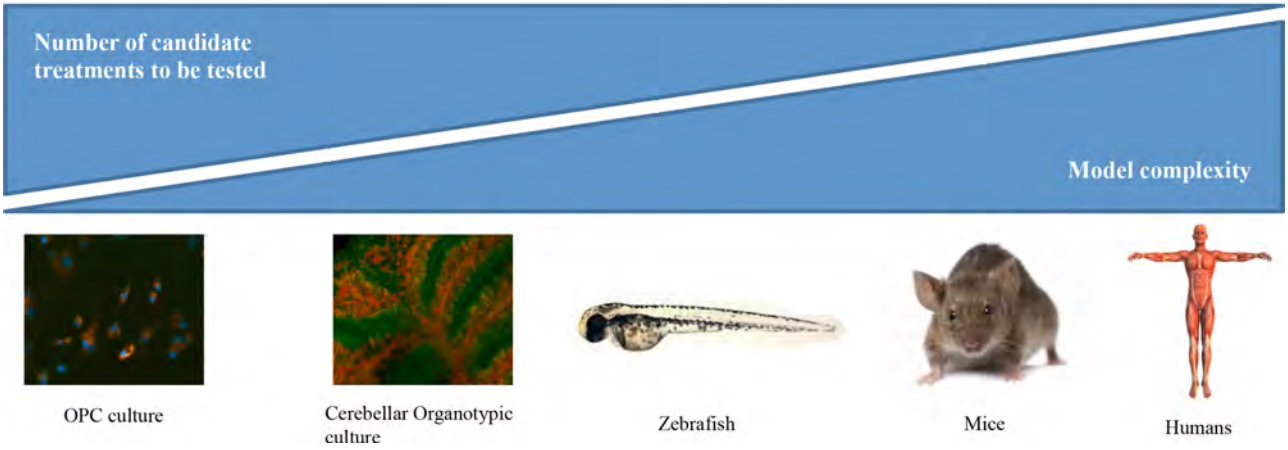

Figure 9: Summary of the models in complexity order and the number of candidate treatments that can be tested in each model. Form left to right: in vitro models (image of OPCs culture), ex vivo models (image of cerebellar organotypic culture), in vivo models (image of Zebrafish and mouse) and at the end of the process, humans.

4. Remyelination therapies

Neurodegeneration is the most incapacitating factor in MS and enhancing remyelination has been postulated as the main strategy to avoid axons to be damaged. Remyelination therapies can be divided in two main strategies, which consist in the transplantation of exogenous pro-remyelinating cells (cell therapies) and the stimulation of endogenous cells to generate new myelin (pharmacological approach).

The idea behind cell therapies is to repopulate the pool of OPC and several efforts have been made in this sense. Oligodendroglial cells demonstrated to remyelinate and restore neurological function in models of CNS demyelination [121,122]. Schwann cells [123], olfactory ensheathing cells or embryonic stem cells have also been postulated as feasible candidates [124]. To continue, transplanted neural stem precursor cells (NSPC) were able to reach demyelinated lesions and produce positive effects [125]. In addition, intravenously administered mesenchymal stem cells (MSC) reduced demyelination, increase neuroprotection and modulate inflammation in the animal model of the disease (reviewed in [126]). However, beneficial effects of NSPC and MSC have been related with the modulation of the microenvironment and with the release of soluble factors respectively, rather than with direct cellular regeneration [127,128].

Although these are interesting results, there are some arguments against the use of cell therapy in MS. First, undifferentiated OPC have been found in MS patients´ lesions being unable to differentiate to oligodendrocytes and to generate new myelin and there is no reason to think that transplanted cells would not be inhibited in the same way. Secondly, it seems logical to think that these therapies could be useful when no OPC are present in the lesions, which seems not to be the case of MS. To continue, intravenous administration has not been effective and direct administration into the lesions is not feasible due to the multifocality of the disease. Finally, we should add the possible ethic limitations regarding the use of some cell types such as embryonic stem cells.

Having these considerations in mind and remembering that OPC differentiation appears to be the most vulnerable step in remyelination failure, an increasing interest in targeting the pathways involved in this process is appearing (pharmacological approach)[127]. The strategies to promote OPC differentiation can be divided in two main approaches; the blocking of remyelination inhibitors or the use of remyelination accelerators [127].

Regarding to the first approximation, for example, extracellular matrix has been shown to inhibit remyelination and the pharmacological manipulation of extracellular matrix can improve remyelination [129]. Interestingly, Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 1 (*LINGO1*) has been

postulated as the most promising remyelination target. *LINGO1* has shown to inhibit OPC differentiation [130] and the inhibition of *LINGO1* has demonstrated to accelerate CNS remyelination in a model of lysolecithin induced demyelination [131–133]. However, humanized monoclonal antibodies against *LINGO1* failed in a phase 2 trial in MS due to poor results[134].

In order to accelerate remyelination, several strategies have been studied. For example, electrically stimulated demyelinated axons form new glutamatergic synapses with OPCs causing OPCs to exit the cell cycle and undergoing differentiation [135]. In addition to this, the activation of RXRγ is able to induce OPC differentiation by binding to thyroid hormone receptor or peroxisome proliferator-activated receptor-γ [136]. Vitamin D has also been identified to regulate OPC differentiation in the RXRγ pathway[137].

Moreover, in the last decade other strategies have also been postulated as feasible remyelination inducers. In the context of this work, the ability of **Extracellular Vesicles** (EVs) and **non-coding RNAs** (ncRNA) to induce remyelination is going to be studied. To understand in deep the role that both factors can play in promoting myelination regeneration, specific sections will address all the related information (sections 5 and 6 of the introduction respectively)

5. Extracellular vesicles and remyelination

Adapted from Sáenz-Cuesta et al (2014) [138] *and Osorio-Querejeta et al (2018)* [139].

Extracellular vesicles (EVs) are membrane-bound particles coming from inside a cell or formed directly from its membrane, and excreted to the extracellular medium. They carry information whose function is cell-to-cell communication without direct contact. They play a role in physiological and pathological conditions, being released during cell activation, stress and apoptosis. Specifically, these vesicles carry proteins, lipids and genetic material such as DNA, mRNA, and ncRNA, producing genotypic [140] and phenotypic [141] modifications in the recipient cell. These interactions are facilitated by the receptors placed on the surface of the EVs membrane, which allow the recognition of the target cell and the vesicles [142].

5.1. EVs classification

5.1.1. Biogenesis

Though there are several ways of classifying EVs, the main division in nomenclature is based on biogenesis. Those formed inside multivesicular bodies and released extracellularly upon fusion of these bodies with the plasma membrane are called exosomes [143]. Their main characteristic is to have a uniform size of between 30 and 150 nm, making them the smallest EVs. On the other hand, those known as microparticles (MP), microvesicles (MV) or ectosomes come from the modification of the cell membrane after external or internal stimuli. This leads to a softening of the membrane-adjacent structure and allows evagination and vesicle formation, which followed by fission on the connecting membrane stalks until their full detachment. These MV/MP vary greatly in size, ranging from 0.3 to 1 µm in diameter [144,145]. However, the size distribution of vesicles is overlapped between groups and sometimes difficult to differentiate, therefore, the International Society of Extracellular Vesicles proposed to call the entire set "extracellular vesicles" [146] (Figure 10).

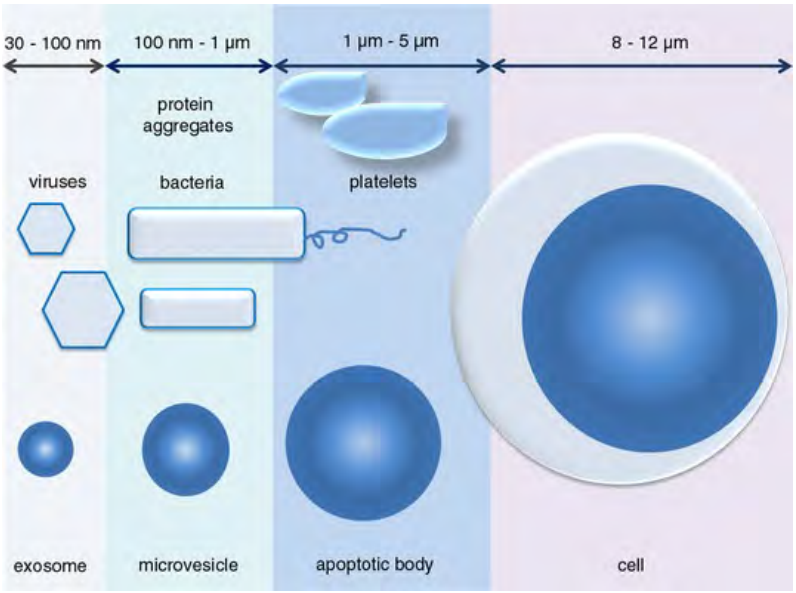

Figure 10: Classification and size of different EVs subtypes. From György, B. et al 2011 [147].

5.1.2. Cell origin

EVs have been also classified in relation with the parental cell from which they arose, being so far those obtained from circulating cells in peripheral blood the most studied

ones. Each cell has characteristic markers on its membrane enabling subsequent identification of the EV, e.g., as erythrocyte-, leukocyte-, platelet-, endothelial- or monocyte-derived. Further, studies focusing on central nervous system (CNS)-derived EVs have described neural stem cell-, neuron-, astrocyte-, microglia-, and oligodendrocyte-derived vesicles [144]. Interestingly, these vesicles can be detected remote from the site of release after cell activation, and have been postulated as markers of the CNS status.

5.2. Techniques for studying EVs

The study of EVs is not straightforward, particularly with respect to isolation and characterization due to their small size and the low concentrations found in human fluids. Although efforts have been made to unify criteria in EV research [146,148–152], they are yet not clearly established, making it difficult to compare studies. Differences derived from centrifugation protocols, fluorochrome labelling and analysis represent unsolved barriers to standardization. Nevertheless, the most widely used techniques can be summarized as follows:

5.2.1. Isolation

One of the main approaches that has been used for isolating EVs from human fluids or culture media supernatants is a series of sequential centrifugation steps. Different purities are obtained depending on the number of steps completed. Briefly, a first centrifugation step at a low velocity (200 to 300 g) separates cells from EVs-containing fluid, which can be further purified or directly pelleted. For a further purification, a second centrifugation must be carried out (at 2,000 to 10,000 g, depending on the fluid or EVs fraction required). Otherwise, EVs can be directly pelleted from the first supernatant (centrifuging at forces of 10,000 up to 100,000 g; ultracentrifugation). Though there are many variations among authors, the first approach to EVs analysis is usually based on the aforementioned steps. As an alternative protocol to obtain EVs, a sucrose gradient can be combined with one of the centrifugation steps.

Another isolation technique is polymeric precipitation (e.g., Exoquick, System Biosciences, CA). The main advantage of this approach is rapid sample processing. However, the low purity obtained and mixing of different EVs subsets makes results difficult to interpret. The extraction of EVs by passing a sample through filters is a

cheap and easy method that can be applied alone or combined with centrifugation. Finally, size-exclusion chromatography and immunoaffinity methods are also commonly used analysis methods.

5.2.2. Characterization

Flow cytometry is a widely employed technique for studying EVs, especially for characterizing EVs origin thanks to the possibility of characterizing the membrane markers of their cell of origin. It is a powerful characterization tool, the process is rapid and the results can be quantified. Its main limitation is poor discrimination under 0.5 μm. However, new high-resolution cytometers can detect particles as small as 0.2-0.3 μm.

Other tools are available to characterize nanoparticles in size and concentration with high resolution. They measure particles based on tunable resistive pulse sensing (qNANO, IZON Science, New Zealand) and Brownian motion of the particle with nanoparticle tracking analysis software (NTA, Nanosight, UK). The simple and user-friendly operation and powerful measurements provided by these instruments have made them reference in the analysis of EVs.

Electron microscopy is usually performed in combination with previous techniques to provide direct evidence of the presence of EVs and morphological information. On the other hand, the expensive and complex processing of samples limits its use.

Fluorescence microscopy is normally used to analyse EV function *in vitro*, as well as to localise EV in tissues and budding process. In particular, confocal microscopy is widely used in EV research. However the lower size of EVs makes the acquisition of the images a tricky question.

Interestingly, in the last year the study of the cargo of EVs is increasing our knowledge about the functions of these vesicles. Enzyme-linked immunosorbent and Western blot assays are being employed for the analysis of EVs; nevertheless they are less extensively used due to the poor characterization and quantification they provide. Notably, expression arrays and next-generation sequencing techniques are currently expanding to the field of EVs, specifically in an attempt to characterize their genetic cargo together with other –omics techniques such as proteomics or lipidomics .

5.3. Why extracellular vesicles?

As it has been previously mentioned, EVs are involved in many biological processes, being their capacity to regulate immune response and cell differentiation the two most important processes in the context of this work [153]. Vesicle secretion and the transfer of material carried within them in the CNS under physiological conditions were described many decades ago [154]. The classic example was the presence of vesicles in the neuronal synapses [155], which was also confirmed recently [156]. It has been observed that EVs are released by neural cells, oligodendrocytes, neurons, microglia, astrocytes in the brain and Schwann cells in the peripheral nervous system (reviewed in [144,157]) transmitting information across de CNS [158]. Finally, they play a role in myelin sheath biogenesis [159,160], as well as in the repair of damaged neurons [161]. All these imply that EVs perform functions necessary for growth and normal functioning of the nervous system.

In addition, EVs are involved in processes of CNS diseases carrying specific pathological cargo or performing functions that produce potential damage [144]. Interestingly, several diseases have been linked to EVs, including neurodegenerative diseases [162,163]. Many studies have found variations in the number and function of circulating EVs in diseases such as Alzheimer's disease, dementia, epilepsy, stroke, traumatic brain injury, malaria, and tumours (mainly glioblastoma), among others (reviewed in [164]). On the other hand, few studies have explored whether variations in EVs in CSF directly reflect the pathophysiology of the CNS [165–171] and only a couple of them have examined EVs derived from the CSF as a surrogate marker for what occurs in the CNS [165,166]. Of particular interest to this work, alterations in the concentrations and cell origin of EVs have been related with MS [138,165,172,173].

The implication of EVs in CNS physiological and pathological conditions and their ability to carry messages from one cell to another suggests that the use of EVs as a drug delivery system or as a treatment, might be an interesting way of targeting and modulating the course of a disease. Moreover, the fact that EVs are able to cross the BBB makes them strong candidates for CNS disease therapy [174].

5.4. Therapeutic potential of EVs for demyelinating diseases

Several works have been published demonstrating the therapeutic potential of EVs. These works will be discussed in the following paragraphs and have been summarized in Table 2.

In some demyelinating pathologies, such as MS, the immune system is responsible for the damage caused to myelin. In this way, the ability of exosomes isolated from pregnant mice serum or human periodontal ligament stem cells to reduce the clinical score of the EAE animal model, has been addressed by inhibiting the immune response[175,176]. In addition, the intranasal administration of curcumin-loaded glioblastoma-derived exosomes to EAE animals ameliorated the clinical symptoms of the model, demonstrating that exosomes could work as anti-inflammatory drug delivery vehicles [177].

As it was mentioned in the introduction, Ols are responsible for generating myelin that enwraps axons. The communication between Ols and axons is essential for the survival and functional maintenance of both. Interestingly, this communication between Ols and axons has been shown to be mediated by exosomes and, in addition, the interactions between Ols and axons might affect the cargo of exosomes [178]. Moreover, when the cargo of exosomes released by Ols was analysed, researchers found that those vesicles contained high levels of myelin related proteins; more concretely PLP, MBP, MOG and CNP [179]. This data was the first evidence of the possible role that exosomes could be playing in myelination. In a more recent work, it was suggested that Ol-derived exosomes were able to inhibit the differentiation of OPCs [160]. Even though the authors did not demonstrate the mechanism by which Ols regulate OPCs in an inhibitory way, these results reinforce the implication of exosomes in OPC differentiation, an essential step for myelination and remyelination. In a different work, the ability of pregnant mice serum-derived exosomes to promote the trafficking of OPCs into lesions after intravenous administration in the EAE was shown [175] emphasizing the implication of exosomes in myelination related processes.

To analyse the role that EVs play in pathological systems, several models have been used. In a model of white matter infarction in rats, researchers demonstrated that EVs

derived from microvascular endothelial cells (MVECs) were taken up by OPCs, inhibiting the apoptosis of OPCs and promoting survival, proliferation and motility of the cells. The authors demonstrated that those EVs contained microRNAs and adhesion molecules which were responsible for the shown effects [180]. Moreover, Mesenchymal Stem Cell-derived exosomes (MSC-Exs) have been shown to promote oligodendrocyte formation and remyelination in a model of subcortical ischemic stroke. After intravenous administration of MSC-Exs, authors were able to detect higher levels of MOG protein and more myelinated axons [181].

Furthermore, a work published in 2014 demonstrated that exosomes from blood of young and environmentally enriched rats significantly increased the myelin content, oligodendrocyte precursor and neuronal stem cell levels and reduced oxidative stress and astrogliosis in demyelinated hippocampal slice cultures [182,183]. They also tested the effect of these blood-derived exosomes in vivo by intranasal administration in aged rats, showing positive results in myelin generation. The authors related the exosomes-derived pro-remyelination effect to their cargo, suggesting that the presence of microRNAs could be responsible for promoting remyelination [182].

Another aspect of demyelinating diseases is that the lack of myelin wrapping axons might, if remyelination does not take place, induce the disruption of the axons and, therefore, neurodegeneration. Neuroprotection is a key factor that might improve patients' outcome and increase their life quality. Regarding to this, mesenchymal stem cells derived EVs were shown to be effective in models of traumatic brain injury after both intravenous or intraperitoneal administration, decreasing inflammation and increasing neuroprotection, angiogenesis and neurological function, opening therapeutic possibilities in which neuroprotection can be reinforced [184,185].

5.5. Delivery into the Central Nervous System

To be able to use EVs as therapeutic biopharmaceuticals for MS, it is imperative to ensure that EVs will reach the CNS. That can be achieved, for example, by delivering EVs directly to the brain, by using systemic injections, or by administering vesicles via intranasal route. The intranasal route can be efficient for different cell type derived EVs, including T-cell, fibroblast and tumour derived exosomes [177]. This delivery route not

only leads to increased brain accumulation of exosomes, but more importantly, it has also shown to reduce inflammation in EAE animals when exosomes are loaded with therapeutic anti-inflammatory molecules, as was previously mentioned [177] . The latter clearly underlines the potential of EVs for treating MS via the intranasal route, which is further supported by successful experiments conducted in the context of other CNS diseases such as Parkinson's disease (PD). In a mouse model of PD, catalase-loaded macrophage exosomes reached the brain and provided antioxidant-mediated neuroprotection [186]. Neuroprotection was also induced by curcumin loaded embryonic stem cell exosomes in an ischemia-reperfusion injury model [187]. Repeated treatments with curcumin-loaded exosomes led to a reduction of inflammation and improved neurological score and restored the expression of several BBB proteins.

Reference	EVs type	EVs Source	Isolation method	Principal experiment	Route of administration	Result
Williams et al., 2013	Exosomes	Virgin and pregnant mice serum	Ultracentrifugation	EAE	Intravenous	Stablished EAE supression.
Rajan et al., 2016	Exosomes	HPLSC culture supernatant	ExoQuick TC	EAE	Intravenous	Inmmunomodulation of EAE.
Zhuang et al., 2011	Exosomes	Glioblastoma culture supernatant	Sequential centrifugation steps	EAE	Intranasal	EAE inhibition.
Frühbeis et al., 2013a	Exosomes	Oli-Neu cultures supernatant	Sequential centrifugation steps	Oligodendrocyte-neuron co-culture	N/A	Exosomes mediated communication.
Krämer-Albers et al., 2007	Exosomes	Primary oligodendrocytes culture supernatant	Ultracentrifugation	Oligodendrocyte culture	N/A	Exosomes contain PLP, MBP, MOG and CNP.
Bakhti et al., 2011	Exosomes	Primary oligodendrocytes culture supernatant	Sequential centrifugation steps	Oligodendrocyte culture	N/A	Oligodendrocytes derived exosomes inhibit OPC differentiation.
Kurachi et al., 2016	Extracellular vesicles	MVECs culture supernatant	ExoQuick TC	Oligodendrocyte Precursor cell culture	N/A	OPCs survival, proliferation and motility.
Otero-Ortega et al., 2017	Exosomes	MSC culture supernatant	miRCURY Exosomes Isolation Kit	Subcortical ischemic stroke	Intravenous	Promotion of olifodendrocyte formation and remyelination.
Pusic and Kraig, 2014	Exosomes	Youth and Environmental Enriched rat serum	ExoQuick TC	Old rats	Intranasal	Enhanced myelin content.
Pusic et al., 2016	Exosomes	Environmental Enriched rat serum	ExoQuick TC	Demyeliantion hipocampal slice culture	N/A	Myelination increased and oxidative stress reduced.
Doeppner et al., 2015	Extracellular vesicles	MSC culture supernatant	PEG precipitation method	Ischemic stroke	Intravenous	Neuroprotection and neuroregeneration.
Drommelschmidt et al., 2017	Extracellular vesicles	MSC culture supernatant	PEG precipitation method	Perinatal brain induced inflammation	Intraperitoneal	Immunomodulation and reduction of micro- and astrogliosis.

Table 2: *Summary of therapeutic potential of EVs for demyelinating diseases*

However, it appears that EV loading with exogenous cargoes prior to intranasal administration is not always essential for therapeutic effects in the CNS, as recently demonstrated in a status epilepticus mouse model. Unmodified human bone marrow derived MSC-Exs reduced neuron loss and inflammation in the hippocampus of treated mice, which more importantly led to preservation of memory function [188]. These properties of unmodified MSC-Exs for treating CNS disease are particularly interesting and promising for MS. Given the trend towards replacing certain MSC cell therapies with EV based therapies, and the fact that a number of MSC cell therapies have been tested in Phase I/II clinical trials for treating MS as well [189], it is likely that MSC EVs will gain further focus in the short term for targeting MS pathology as well.

In addition to the intranasal administration route, as described above, other local delivery options have shown efficacy for EV based CNS therapies as well. Unilateral direct brain infusion of glioblastoma derived exosomes, pre-loaded with hydrophobic small interfering RNA (siRNA), led to exosome-dependent bilateral Huntington messenger RNA (mRNA) silencing in the brain of treated mice [190]. Other therapeutic strategies not directly relying on drug delivery can be efficient as well. Intracerebral neuroblastoma exosome administration to an Alzheimer disease mouse model reduced amyloid-β levels in the brain and lowered the associated synaptotoxicity, tapping thus into natural EV-mediated Aβ clearance pathways [191]. Similar effects were observed also when using primary neuron exosomes, the effect being cell type specific as glial exosomes were less efficient in the capture of amyloid-β [192]. This is not surprising as the transport of exosomes to brain parenchyma can be specifically related to the presence of specific surface molecules such as folate receptor α [193] as well as other EV related signatures that can, for example, mediate periphery-brain signalling in inflammation [194].

In many cases, however, systemic rather than local therapeutic EV administration would be preferred for various reasons, including the safety of the treatment administration. Despite the fact that BBB has been proposed as virtually impermeable to most molecules there is some evidence that unmodified exosomes can enter the brain to some extent [195], but brain exposure is significantly increased when using certain brain targeting ligands such as the rabies virus glycoprotein derived RVG peptide [196]. The brain targeting RVG peptide, even though the precise targeting mechanism has not been fully elucidated, led to increased brain delivery of siRNA when decorated on dendritic

cell exosomes [197]. Using that strategy, it was possible to lower the levels of a target gene on both mRNA and protein levels in the brains of wild type mice [197,198].

6. Non-coding RNAs as remyelination mediator in MS

Adapted from Osorio-Querejeta et al. (Book chapter in "Design and development of novel therapeutics agents for multiple sclerosis". RSC drug discovery series. To be published in 2019)

Non-coding RNAs (ncRNA) have been related with MS in many characterization studies in which different expression levels of small ncRNA has been described between MS patients and healthy controls and also between different classes of MS. In this context, the main established relation between sncRNA and MS has been through the potential interest of these molecules as biomarkers in MS [199]. However, a functional role for this sncRNA in the etiopathology of MS has been also postulated.

MicroRNAs (miRNAs), a type of sncRNA, are short sequences of RNA (21-24 nt) that regulate gene expression by binding to target messenger RNAs (mRNA) and preventing their translation to protein. Canonical microRNAs are transcribed by RNA polymerase II as part of a longer transcript called pri-miRNA. This primary transcript acquires a hairpin secondary structure that is processed by Microprocessor, a protein complex formed by one molecule of the Drosha endonuclease and two molecules of DGCR8. Drosha cuts at the base of the hairpin of the pri-microRNA to generate a shorter RNA of about 60 nucleotides-long. This stem-loop structure called pre-miRNA is transported to the cytoplasm by Exportin-5 [200], where will further be processed by Dicer. This endonuclease will generate a miRNA duplex after cutting both strands near to the loop. Finally, this miRNA duplex is loaded into an Argonaute protein and only one of the two strands is kept in the complex, which will be the functional while the other strand is usually degraded. Once the microRNA is loaded into the silencing complex, it binds to the target mRNA by base-pairing and either produces its degradation or inhibits its translation to protein [201].

In animals, microRNA-mediated repression requires the binding of the protein TNRC6, that recruits other proteins which depending on the context, cause mRNA decay or

translation repression[202]. The predominant way of microRNA-mediated regulation of transcription is through targeting mRNA [203,204], which means that the effects of microRNA regulation can be measured at mRNA level.

It has been described that miRNA take part in virtually all biological functions such as development, cell differentiation, proliferation, cell death and cell signalling. Taking into account that they are part of the gene expression regulation network, it is not surprising that the alteration of these small molecules has an impact on human health. In fact, an increasing number of articles are being published describing the relationship of microRNA deregulation with several human diseases such as cancer, cardiovascular diseases, neurological disorders and immune mediated diseases[205–209].

The first evidence of the implication of microRNA in multiple sclerosis was reported in 2009 by three groups [210–212]. Otaegui and colleagues reported that miR-18b and miR-599 were overexpressed in leucocytes from MS patients during relapse episodes. Moreover, they proposed that miR-96 was relevant for remitting phase of the disease, which was identified by co-expression networks[210]. Short after having described the deregulation of some microRNA in leucocytes from MS patients, another group reported the dysregulation of 165 microRNA in MS patients compared to healthy controls and they found that miR-145 was able to correctly classify 89.7% of the samples tested (a total of 39) [211]. Interestingly, in an attempt to improve these results, Keller and colleagues apply machine-learning techniques to select a subset of microRNA, which was able to distinguish both groups. This analysis yielded a group of 48 microRNA able to discriminate patients from healthy controls with an accuracy of 96.3%, a specificity of 95%, and a sensitivity of 97.6%.

After these first profiling studies, several studies have been published trying to elucidate the specific role of miRNA in multiple sclerosis. Those works, have analysed microRNA expression in different sample types in humans (leucocytes, serum, plasma, specific lymphocyte subsets, CSF and brain) and also in different tissues of the EAE model. These works have provided a list of miRNA that can have a role in multiple sclerosis by regulating different processes in the immune cell development and differentiation as well as in neurodegeneration and remyelination.

MicroRNA have been the most widely studied family of sncRNA, but other types, such as small nucleolar RNA (snoRNA) have also been related to MS. snoRNA are grouped into two main families: C/D box and H/ACA box snoRNA, according to the evolutionarily conserved sequence elements[213] (Figure 11).

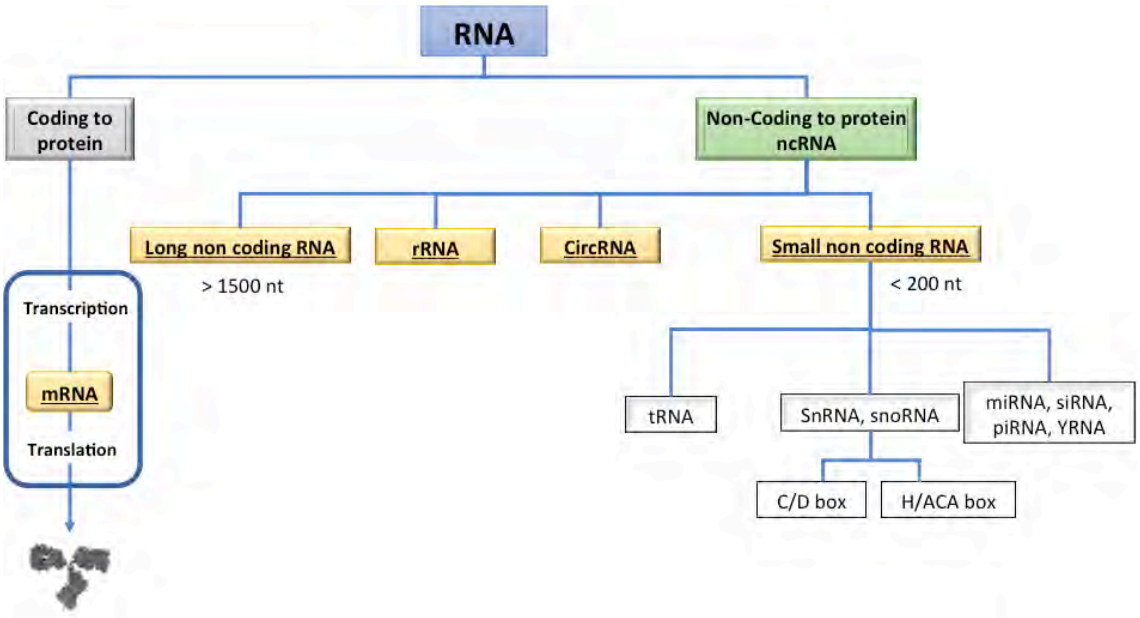

Figure 11: Simplified scheme of RNA classificaition.

The risk of developing multiple sclerosis has been, in part, associated with epigenetic modifications in which ncRNAs are implicated [214]. In this sense Drosha, Dicer and DGCR8 have been found to be overexpressed in RR MS patients, underlining the possible involvement that the dysregulation of microRNAs might play in the pathogenesis of MS [215]. In addition to this, long non-coding RNAs, which also regulate gene expression, have been associated with the regulation of OPC differentiation and the development of MS animal models [216]. Although long non-coding RNAs (lncRNAs) as therapeutic targets in epigenetic diseases has been proposed [217], their functions and potential as therapeutic mediators remain largely undefined [218] and no papers have been published elucidating the role that this kind of ncRNAs may play in MS therapy. On the other hand, microRNAs have been postulated as feasible and promising molecules to induce both immunomodulation and remyelination (the two main goals in MS therapy).

6.1. NcRNA as Immunomodulators in MS therapy

Several strategies have been used to identify potential targets and mediators for immunomodulatory ncRNA mediated therapy. One of these consists in the use of expression networks analysis, which has been used to identify dysregulated ncRNAs in patients, both in relapse and remission, and in controls. This kind of approaches can led to a list of therapeutic ncRNAs candidates [219]. It has been shown that this dysregulation favoured proinflammatory and T cell mediated autoimmunity, highlighting the role that microRNA play in the development of the disease and opening therapeutic approaches in microRNA mediated immunotherapy for MS [220].

Evaluation of microRNAs expression has been used as biomarkers of treatment efficiency and disease evolution given that different drugs affect miRNA expression. However, these studies have also been used to determine microRNAs that can be targets of immunomodulatory therapies.

The dysregulation of microRNAs founded in MS (and in different stages of the disease) opened the opportunity to the discovery of new therapeutic targets [221]. For example, miR-141, miR-200a and miR-448 have been found to be up-regulated in MS patients. These microRNAs regulate Th17 cell differentiation while inhibiting Treg differentiation [222,223]. Polarization of T cells to Th1, Th2 or Th17 is a critical process in cell mediated immunity and these microRNAs could be the targets for an immunomodulatory therapy in MS patients. Related with this, treatment of patients derived T-cell with microRNA inhibitors for miR-27, miR-128 and miR-340, which are overexpressed in MS patients, were able to restore Th2 cytokine production *in vitro* [220]. Moreover the role of microRNAs as immunoregulators has also been addressed *in vivo.* MiR-155 is a positive regulator of CNS autoimmune inflammation and miR-155 knock out mice generated a less severe EAE by decreasing Th1 and Th17 response in the CNS. In addition, when EAE WT animals were treated with locked nucleic acied-modified anti-miR-155 mice clinical severity was reduced [224]. Moreover, the lentiviral infection of miR-326 in EAE mice, a microRNA which is up-regulated in MS patients, leads to an increase in disease severity by targeting ETS-1, a known negative regulator of Th17 cells [212]. These results indicate that miR-155 and miR-326 may be potential targets for MS patients. Finally the down-regulation of miR-106a-363 cluster and the

up-regulation of miR-132 have shown to lead to more severe EAE evolution and therefore they have been proposed as a therapeutic target for anti-inflammatory treatments in MS [225,226]

Taking all this information together, we can summarize that microRNAs are active players in immune alteration that take place in MS and therefore their utilization as mediators or targets in future MS therapies is a promising field that is giving its first steps.

6.2. NcRNAs as remyelination promoters

It has been widely proposed, as explained previously, that the stimulation of OPC differentiation could increase myelin regeneration being a promising therapeutic approach to induce remyelination in MS. As it will be discussed below, microRNAs play an important role in the precise and complex program that take place in OPC differentiation and remyelination (Table 3).

Dicer knock out mice showed impaired myelination, which was related with a poor OPCs differentiation, as the observations of proliferating OPCs but the lack of oligodendrocytes suggested [227,228]. Dicer 1 and Olig 1 knock out mice were used to search for the microRNAs responsible for oligodendrocyte maturation. miR-219 and miR-338 expression were significantly reduced in both animals indicating the role that these microRNA may be playing in OPCs differentiation. In addition to this, inducible oligodendrocyte Dicer knockout mice generate demyelination and inflammatory gliosis. Interestingly, these oligodendrocytes reduce the production of miR-219, highlighting the importance of this microRNA [229]. In this sense, differentiating OPCs and human white matter have been shown to be enriched in miR-219 and conditional miR-219 mutant mice showed reduce numbers of OL in the corpus callosum and optic nerve but not lower OPCs levels [230,231], suggesting that miR-219 is necessary for OPC differentiation. To reinforce this idea, OPCs were obtained from a Dicer 1 knock out mouse being unable to differentiate to Ol. However, when miR-219 was transfected to these cells, they increased the expression of MBP proteins and the overall expression of CNP, MBP and MOG genes, which are associated with oligodendrogenesis [228]. In addition, human endometrial-derived stromal cells have been shown to differentiate to

pre-oligodendrocytes after lentiviruses mediated miR-219 overexpression [232]. Similar to this, mouse embryonic stem cells transfected with miR-219 were able to differentiate to OPCs, and induced remyelination more efficiently than wild type OPCs after their transplantation into a mice model of toxin demyelination [233]. In the same model of cuprizone induced demyelination, lentivirus overexpressing miR-219 were intrathecally administered, increasing MBP and CNP levels and decreasing demyelination in the model [234]. In addition, miR-219 inducible knockout mice were demyelinated by injection of lysolecithin and fewer remyelinating sings were detected indicating that miR-219 is critical for remyelination after a demyelinating insult. In order to confirm these results, miR-219 overexpressing mutant mice were generated and lysolecithin induced demyelination generated in the spinal cord. These animals were able to generate more OPCs and to increase the percentage of remyelinated axons [230]. Finally, when mimic-219 was administered intrathecally to the EAE animal model, a decreased in the clinical score was shown thanks to the generation of new myelin forming oligodendrocytes [230]. These results suggest that the elevation of miR-219 induces OPCs differentiation and therefore remyelination, opening a promising therapeutic approach.

Mir-219 has been predicted and experimentally demonstrated to target *PDGFRα*, *Sox6*, *FoxJ3* and *ZFP238*, all of which are related with OPCs differentiation [228]. The expression of *PDGFRα* induces OPC proliferation and *Sox6* prevents their differentiation. Another proposed route of action for miR-219 is related with the repression of Lingo1 expression, a protein that has demonstrated to inhibit OPC differentiation.

Although miR-219-5p is the most promising candidate to show remyelinating potential, is not the only microRNA described to be involved in these processes. Both, miR-219 and miR-338 are overexpressed in perinatal stages coinciding with oligodendrocyte maturation [227] and miR-338-5p appears also to be upregulated during mouse brain maturation and to regulate OPC differentiation in humans [231]. Moreover, the overexpression of miR-338 in human endometrial stromal cells by lentivirus induce their differentiation to OPCs [235]. Interestingly, miR-338 and miR-219 knock out mice did show lower myelin levels when compared to miR-219 knock out mice, indicating that miR-338 cooperate with miR-219 in the regulation of oligodendrocyte maturation

[230]. As a consequence, the combination of both microRNAs has seen to be more effective inducing OPC differentiation *in vitro* [76,78].

MiR-146a has also been proposed to regulate this process. Primary OPCs culture transfected with miR-146a differentiate to oligodendrocytes whereas the inhibition of this microRNA with hairpin inhibitors block their differentiation [236]. This microRNA has shown to be up-regulated during the first stages of cuprizone induce demyelination model [237]. In addition, the infusion of miR-146a in the model stimulates the generation of oligodendrocytes and augments myelination [237], which has been related with the role that the microRNA might play in myelin restoration. This effect has shown to be mediated by Interleukin-1 receptor-associated Kinase 1 (IRAK1) [236]. Nevertheless, these results are somehow in contrasts with latter ones that showed higher numbers of oligodendrocytes and reduced demyelination in a miR-146a knock out mice[238]. Mir-146a has also been described to reduce the inflammatory response, and this might explain these contradictory results [239].

To finish with the use of microRNAs as remyelination inductors, the overexpression of miR-23a in OPCs leads to an increase in CNP positive cells. Genetically engineered mice to overexpress miR-23a not only increase myelin related genes expression, but also myelin thickness in the corpus callosum [240]. Interestingly, miR-23a has shown to up-regulate 2700046G09Rik, a lncRNA which increases the half-life of miR-23a. The presence of this lncRNA in oligodendroglia has been proposed to potentiate the activation of miR-23a/PTEN/Akt/mTOR and MAPK cascades regulating the expression of myelin related genes in Ols [241].

MicroRNAs has also been proposed to be potential target of remyelinating drugs. miR-221-3p was proven to inhibit Schwann cell myelination in vivo by targeting NAB1 [242]. Similar to this, miR-212 and miR-297c-5p were shown to inhibit the maturation of Ols [243,244]. In addition, the overexpression of miR-125a-3p has been described to impair rodent oligodendroglia maturation. More precisely, this microRNA reduces the number of MBP positive oligodendrocytes. Although MBP is not a predicted target of miR-125a-3p, by *in-silico* analysis tools this microRNA has been reported to regulate myelination pathways up-stream. Interestingly, miR-125a-3p has been shown to be up-regulated in MS patients´ CSF, which could indicate that this microRNA is inhibiting

remyelination[245]. Although the role of these microRNA in central nervous system remyelination should be studied deeply, its down-regulation may be a therapeutic approach to stimulate oligodendrocyte differentiation and remyelination. To conclude, miR-26a, a microRNA that is overexpressed on IFN-β treated patients, targets SLC1A1 which is involved in glutamate receptor signalling pathway. Excessive glutamate is released in demyelinating lesions and this can be a cause of neuron toxicity [246]. Targeting miR-26a can promote neuroprotection or at least inhibit neuron-toxicity in neurodegenerative diseases [247].

6.3. ncRNA delivery to the CNS

NcRNAs have shown to play a role as immunomodulators and myelin inductor making them therapeutic candidates for MS. The use of sncRNAs and more concretely microRNAs as therapeutic mediators may involve the manipulation of microRNAs levels by increasing or repressing their expression in the affected tissue. This could be done by miRNA mimic (agonist) or by anti-miR (antagonist) increasing or decreasing target genes expression respectively [248]. In order to perform an effective and safety microRNA administration, two main considerations should be made: the route of administration and the delivery method.

Regarding to the first question, microRNAs have been shown to be effective mediators in the CNS after direct administration in animal models [230]. Nevertheless, direct administration by intrathecal injections should be avoided in human therapy, especially if repetitive administrations have to be made, therefore intravenous, intranasal or intraperitoneal administration have been studied as therapeutically approaches. However, the CNS in protected by the blood brain barrier (BBB) and delivery of microRNA or their repressors to the CNS is a challenging question. In addition, nucleic acids can be degraded by enzymes and they must be assisted to entrance into the target cells avoiding the risk of being taken by non-target tissues up. To cover all these, microRNAs or their antagonists should be encapsulated to protect them and to cross the BBB and reach the CNS to produce the expected effect.

In relation with this, several are the delivery methods that have been studied. These go from viral vector such as adenovirus, retrovirus or lentivirus to synthetic nanocarriers.

Viruses have demonstrated to be effective delivering microRNAs to the brain. However, safety questions should be taken into account such as a possible oncogenic transformation of the recipient cell. In addition, this delivery method can stimulate the innate and adaptive immune responses reducing its effectivity [249]. On the other hand, synthetic nanocarriers have been appeared as promising microRNAs vehicles due to the known composition, easy to manage and analysed and lower immunogenicity [250]. Several are the delivery systems to be used, standing out liposomes and nanoparticles. These have been shown to be able to cross the BBB and delivery a specific drug or molecule to the CNS [251]. In addition, their surface can be bounded to a concrete ligand making them specific for a cell type or tissue. Interestingly, in the last years a new microRNA delivery method has been appeared which is a middle step between virus vectors and synthetic nanocarriers. This consists in the use of extracellular vesicles.

As has been addressed before, EVs are 100 to 1000 nm size vesicles released constitutively by cells and that play an important role in intercellular communication by delivering proteins and genetic material, among others [252,253]. They have been proven to cross the blood brain barrier [177], to take part in the transmission of information across the CNS [158] and to be a potential therapeutic methods for demyelinating. Interestingly, young rats derived EVs have shown to be able to induce myelin formation in aged rats. This effect was related with the microRNA cargo, and more concretely with the presence of miR-219 in the vesicles, reinforcing the role that EVs may play in microRNA delivery to the CNS for the treatment of MS.

microRNA	Role in MS	Status	Therapeutic Strategy	Expected Result	Reference
miR-16	Autoimmunity control	Up-regulated	Antagonist	IS regulation	Arruda *et al.,* 2015
miR-23a	-	-	Agonist	increase myelin tickness	Lin and Fu, 2009
miR-26a	Glutamate levels increase	Up-regulated in IFN-B treated patients	Antagonist	Neuroprotection	Potenza *et al.,* 2018
miR-106a-363 cluster	Inflammation and BBB breakdown	-	Agonist	IS regulation	Ingwersen *et al*., 2015
miR-125a-3p	Oligodendroglia maturation inhibition	Up-regulated	Antagonist	Oligodendrocyte maturation	Lecca *et al*., 2016
miR-141	Th17 and Treg differentiation regulator	Up-regulated	Antagonist	IS regulation	Naghavian *et al*., 2015; Huang *et al*., 2016; Wu *et al*., 2017
miR-142-3p	Regulation of inmuno tolerance	Donw-regulated	Agonist	IS regulation	Waschbisch *et al*., 2011; Arruda *et al*., 2015
miR-146a	Regulation of inmuno tolerance	Donwregulated	Agonist	IS regulation	Boldin *et al*., 2011; Waschbisch *et al*., 2011
	Inhibition of oligodendrogenesis			Oligodendrogenesis and myelination	Liu *et al*., 2016; Zhang *et al*., 2017
miR-155	Positive autoimmunity regulator	Up-regulated	Antagonist	IS regulation	Murugaiyan *et al*., 2011
miR-200a	Th17 and Treg differentiation regulator	Up-regulated	Antagonist	IS regulation	Naghavian *et al*., 2015; Huang *et al*., 2016; Wu *et al*., 2017
miR-212	Oligodendrocyte maturation inhibitor	-	Antagonist	Oligodendrocyte maturation	Wang *et al*., 2017
miR-219	OPC differentiation promotor	Up-regulated in OPC differentiation	Agonist	OPC differentiation/Remyelination	Shin *et al*., 2009; Dugas *et al*., 2010; Zhao *et al*., 2010; de Faria *et al.*, 2012; Ebrahimi-Barough *et al*., 2013a; Fan *et al*., 2017; Liu *et al*., 2017; Wang *et al*., 2017
miR-221-3p	Myelination inhibitior	-	Antagonist	promote myelination	Zhao *et al*., 2018
miR-297c-5p	Oligodendroglia maturation inhibition	-	Antagonist	Oligodendrocyte maturation	Kuypers *et al*., 2016
miR-326	Th17 cells negative regulator	Up-regulated	Antagonist	IS regulation	Du *et al*., 2009
miR-338	OPC differentiation promotor	Up-regulated in OPC differentiation	Agonist	OPC differentiation/Remyelination	Shin *et al*., 2009; Zhao *et al*., 2010; de Faria *et al*., 2012)
miR-448	Th17 and Treg differentiation regulator	Up-regulated	Antagonist	IS regulation	Naghavian *et al*., 2015; Huang *et al*., 2016; Wu *et al*., 2017

IS: Immune Sistem
OPC: Oligodendrocyte Precursor Cells

Table 3: Summary of the proposed druggable microRNAs for MS

JUSTIFICATION

The development of remyelination therapies is nowadays a priority in MS treatment. Due to the lack of this kind of therapeutic approaches, alternative strategies are being proposed. The work published by Pusic et al in 2014 [182] was, in our opinion, a solid step in this direction. In brief, they demonstrate that young rat derived exosomes were able to induce remyelination in aged rats thanks to the presence of miR-219a-5p in the cargo of these exosomes. However, the analysis of the ability of these exosomes to induce remyelination in a pathological animal model was needed. *"All this considered, this work began in 2013…*

HYPOTHESIS

- Young rodent blood-derived exosomes were described to induce OPC differentiation and myelination in vitro and in aged rodents due to the presence of miR-219a-5p in their cargo. We hypothesised that these exosomes are able to induce OPC differentiation and remyelination in the Experimental Autoimmune Encephalomyelitis model.

- The generation of a new scoring system for monitoring the Experimental Autoimmune Encephalomyelitis model will help and facilitate the acquisition of data and the reproducibility of the experiments.

- If the pro-remyelinating effect shown in young rodent blood-derived exosomes was related with miR-219a-5p, we hypothesised that MS patients will increase miR-219a-5p levels after relapse.

- The non-invasive delivery of miR-219a-5p to the CNS will decrease the clinical manifestation of the Experimental Autoimmune Encephalomyelitis model.

OBJECTIVES

This work has moved forward during its development and new objectives have been appearing during this period, all of which have tried to understand previous steps. We have focused on the following:

- To test the ability of young mice blood-derived exosomes to induce remyelination in a pathological animal model of demyelination (Chapter One).
- To determine the ability of microRNA-219a-5p to induce OPC differentiation and remyelination (Chapter One).
- To generate a new measurement method to analyse the clinical evolution of the EAE animal model (Chapter Two).
- To characterise the level of miR-219a-5p in MS patients in relapse and remission (Chapter Three).
- To find and elucidate a non-invasive and efficient microRNA-219a-5p delivery system to promote remyelination in the Central Nervous System (Chapter Four).

CHAPTER ONE: MiR-219a-5p is a key factor in the cargo of exosomes to induce remyelination.

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(Submitted)

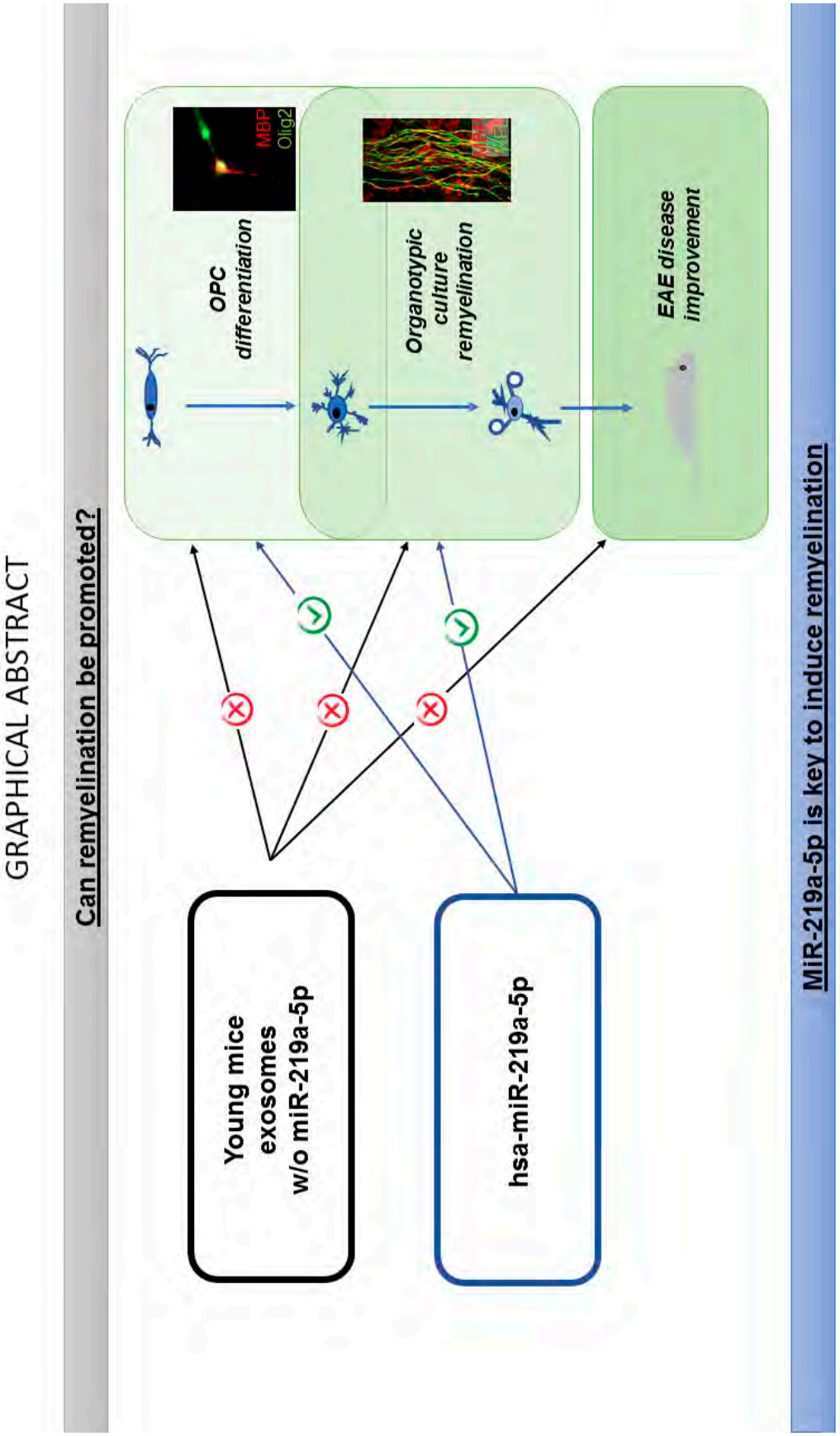

Figure 12: Graphical abstract

INTRODUCTION

Multiple sclerosis (MS) is a central nervous system disease in which myelin is damaged by autoimmune attacks (known as relapses), producing demyelination plaques that are responsible for the clinical symptoms [19,254]. During the first stages of the disease, the organism has the ability to repair these lesions by recruiting OPCs that migrate, differentiate to OLs and produce new myelin that wraps axons [41]. This process, called remyelination, restores axons´ homeostasis and the transmission of action potentials [179,255,256]. However, with the progression of the disease, the remyelination ability declines and the disease enters to a chronic phase in which neurodegeneration is the main feature.

Currently, there is a wide range of treatments for MS that immunomodulate or immunosuppress the immune system, decreasing, and in some cases even avoiding, the number of relapses. Unfortunately, there is no commercially available treatment to promote remyelination and/or neuroprotection. Neuroprotection and neurorepair are the next challenge in MS and the administration of an immunoregulatory treatment together with a remyelination therapy has been proposed to prevent relapses and neurodegeneration. Therefore, alternative strategies are being tested to promote remyelination. One of them consists in the use of exosomes [183,257].

Exosomes are 30-150 nm particles, formed inside multivesicular bodies and released to the extracellular environment upon fusion of these bodies with the plasma membrane [143]. Exosomes are cell-to-cell communication mediators; responsible for carrying information (in the form of proteins, lipids, genetic material and metabolites) that can modify the receptor cell [253]. Exosomes are involved in the pathogenesis of multiple diseases [138], including neurodegenerative diseases. [162]. In the case of MS, for example, exosomes have been related to the regulation of myelin membrane biogenesis [159,160] and the reparation of damaged neurons [161]. Due to this, exosomes are being postulated as possible therapeutic agents. In this sense, Pusic and collaborators published in 2014 a work demonstrating that the administration of exosomes isolated from blood of young mice to hippocampal slice cultures promoted the generation of new myelin. Moreover, they tested the effect of these young rodent blood-derived exosomes in vivo, by

intranasal administration of these exosomes in aged rats, showing positive results in myelin generation [182].

Due to the lack of treatments to promote remyelination in MS and taking into consideration previous results, such as the mentioned above, we attempted to go a step further by studying the remyelination potential of young rodent blood-derived exosomes in a pathological animal model. Under the hypothesis that young rodent blood-derived exosomes might be able to promote OPCs differentiation and therefore remyelination, we generated a MS animal model in which the remyelination potential of young rodent blood-derived exosomes was tested.

RESULTS

1. Exosomes characterization

Nanoparticle Tracking Analysis (NTA) was used to characterize the size and distribution of isolated vesicles. We found two peaks of vesicles of 160 nm and 396 nm. The mode and the mean were 159.4 nm and 185.5 nm respectively. These results indicate a normal Extracellular Vesicles (EVs) distribution (*Figure 13*, A). Western Blot indicated higher concentrations of exosomes protein markers CD71 and CD107a and lower levels of the negative control protein (endoplasmic reticulum 78 glucose regulated protein; Grp78) in exosome sample compared to whole cellular extract (*Figure 13*, B). These results indicate the accuracy of EV isolation approach.

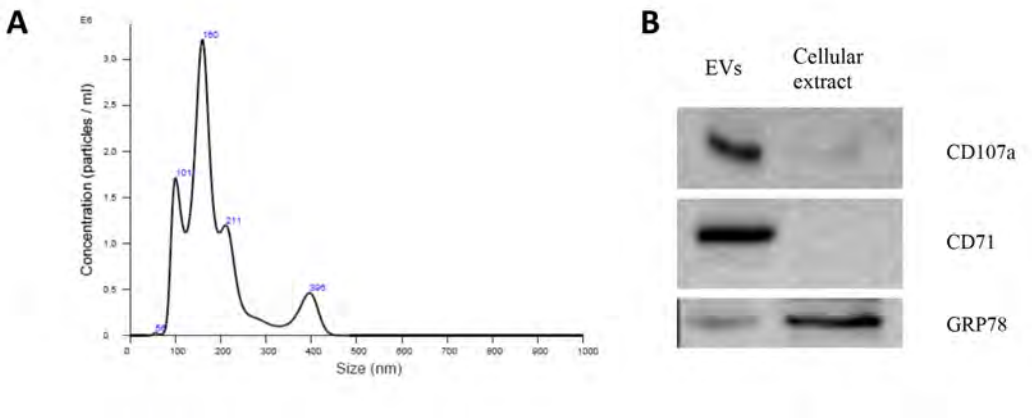

Figure 13: Exosomes characterization: A) Size profile characterization by NTA of a representative sample. Results showed a typical extracellular vesicles distribution with two peaks at 160 nm and 396 nm, a mode of 159.4 nm and a mean of 185.5 nm. B) WB of extracellular vesicles and whole cellular extract, which was used as a control of the purity of the vesicles sample. Results revealed higher concentrations of exosomes protein markers CD71 and CD107a in EVs sample when compared to the control, indicating that the sample was enriched in exosomes. In addition, lower levels of the negative control protein (endoplasmic reticulum 78 glucose regulated protein; Grp78) were shown in exosome sample compared to whole cellular extract indicating that exosome sample did not contain cells (images obtained from different gels and combined into a single image; full images in supplementary material section).

2. Young mice blood-derived exosomes do not ameliorate EAE clinical symptoms

To test the ability of young- mice blood-derived exosomes to promote remyelination in the MS animal model, exosomes were administered intranasally at the disease onset. Three groups were analysed (n = 6 each), treated with young mice blood-derived exosomes at concentrations of 100 μg of protein, 200 μg of protein and a control group (PBS), following the previous protocol of Pusic et al. Clinical score and weight were measured daily showing no significant differences between groups ($p = 0.5$ for DPBS vs 100 μg and $p = 0.15$ for DPBS vs 200 μg) (*Figure 14*, A and B). In order to analyse if exosomes were able to immunoregulate and prevent, or at least delay the appearance of the clinical symptoms, a second experiment was performed in which exosomes were administered 4 days after the disease induction at a concentration of 100 μg. However, no significant differences were observed ($p = 0.42$) (*Figure 14*, C and D).

3. Young mice blood-derived exosomes do not promote remyelination in a cerebellar organotypic culture

To study exosome-mediated remyelination independently of other processes that can be present in the EAE model and might affect the ability of young mice blood-derived exosomes to promote remyelination, we decided to test the remyelination potential of exosomes in a cerebellar organotypic culture, which is a less complex model. Organotypic culture were demyelinated with lysolecithin and treated with several concentrations of exosomes and the vehicle (DPBS) as a control. After three days in culture, confocal images for neurofilament (NFL) and myelin basic protein (MBP) revealed no significant differences in Manders´ colocalization coefficient values between groups and 20 μg of exosomes was stablished as the representative group for treated samples (Manders´ colocalization coefficient values: 0,72 +/- 0.9 for DPBS and 0.78 +/-0.05 for 20 μg of exosomes) (*Figure 15*, A, B and C). To ensure that the model was working properly, the lysolecithin demyelinating effect was measured, showing lower Manders´ colocalization coefficient in lysolecithin treated slices when compared to non-demyelinated cultures concluding that lysolecithin induced demyelination (*Figure 15*, D).

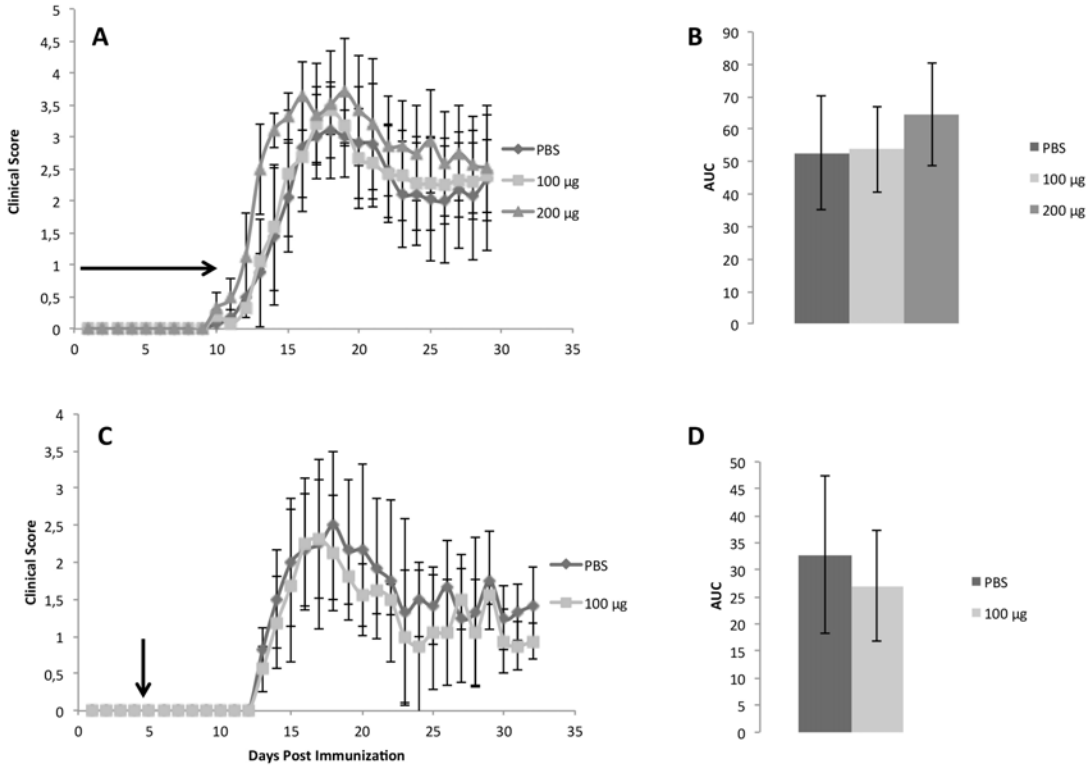

Figure 14: Young mice-derived exosomes do not ameliorate EAE clinical symptoms; A) Clinical score averages of each group (n = 6) are shown. The treatments (100 and 200 µg of exosomes and DPBS) were administered at the disease onset; score = 1 (arrow). B) Mean of area under the curve (AUC) for each group shown in A and their standard deviation. C) Clinical score averages of each group (n = 3 for DPBS and n = 4 for 100 µg of exosomes) are shown. The treatments (100 µg of exosomes and DPBS) were administered 4 days after EAE induction (arrow). D) Mean of AUC for each group shown in C and their standard deviation.

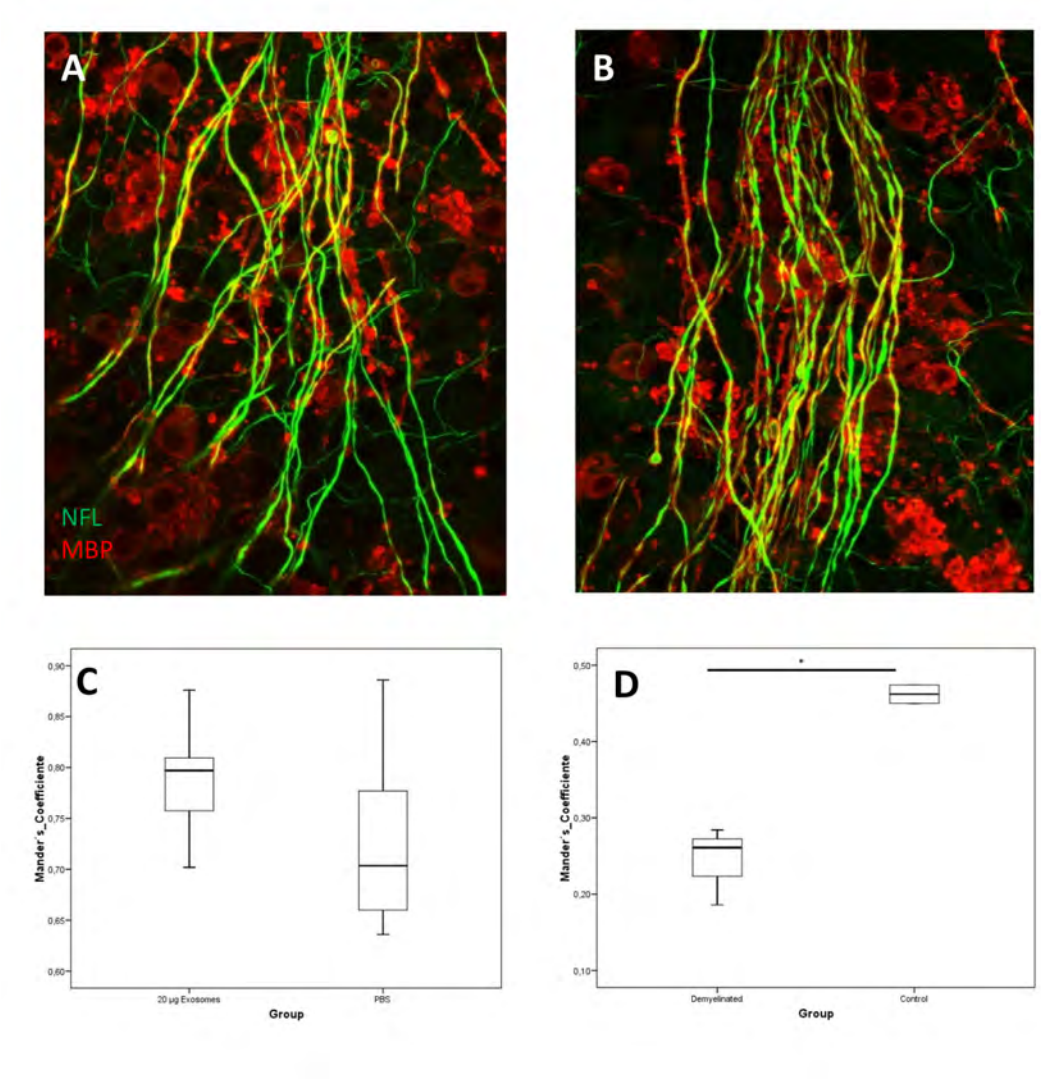

Figure 15: Young mice-derived exosomes do not promote remyelination in a cerebellar organotypic culture: Organotypic culture remyelination analysis. Cultures were demyelinated with lysolecithin for 15 hours after which fresh media was added and treatment performed. Cultures were grown for 72 hours. A and B are representative areas of treated (20 µg of exosomes) and non-treated (DPBS) organotypic culture slices, respectively. NFL is stained in green and MBP in red. Both images are maximum intensity projections C) Manders´ colocalization coefficients of both treated and non-treated samples (3 slices per group; three images per slice were acquired) demonstrating that young derived exosomes are not able to increase remyelination in the organotypic culture. D) Cultures were demyelinated with lysolecithin for 15 hours after which fresh media was added and the culture was allowed to grow for 36 hours. Manders´ colocalization coefficients for cultures treated with lysolecithin and untreated controls are shown, demonstrating that lysolecithin induced demyelination in the cerebellar organotypic culture.

4. Young mice blood-derived exosomes do not promote OPC differentiation

Even though there was no decrease in the EAE score and no increase of remyelination in the cerebellar organotypic culture, we tested whether exosomes could promote OPC differentiation, a necessary step for remyelination. Three different concentrations of young mice blood-derived exosomes and the vehicle without exosomes were administered to the OPC media (0.5, 5 and 50 μg of protein) and RNA was extracted at 2 time points (1 and 5 days after treatment). Gene expression analysis did not show any significant difference between the treated and untreated group at any time points and concentration (*Figure 16*, A and B). To confirm that OPCs were able to take exosomes up, exosomes were labelled with DiI Celltracker and administered to the cell culture. After 4 hours, 72% of the OPCs were stained, showing exosomes integration (*Figure 16*, C).

5. miR-219a-5p is not present in young- mice blood-derived exosomes

To determine if the absence of the remyelination effect shown in our models was due to the lack of pro-remyelinating microRNAs in the young mice blood-derived exosomes, their cargo was analysed. To determine which microRNAs were present in the exosomes, a GeneChip miRNA 4.0 Array was carried out. 465 microRNAs were detected in our samples some of which have been proposed as endogenous references in plasma and serum samples (miR-146a, miR-16, miR-195, miR-30e and miR-744 miR-109 and miR-191) [258,259]. However, miR-219a-5p and none of the miRNAs proposed as remyelination promoters were detected (miR-138, miR-338, miR-9, miR-23, miR-19b and miR-146a). Furthermore, the absence of miR-219a-5p was confirmed by qPCR *Figure 16*, D). As previous experiments by other authors were performed in rats, we also isolated and analysed miR-219a-5p content in rat-derived exosomes by qPCR. In contrast to previous reports, our rat-derived exosomes did not have miR-219a-5p in their cargo.

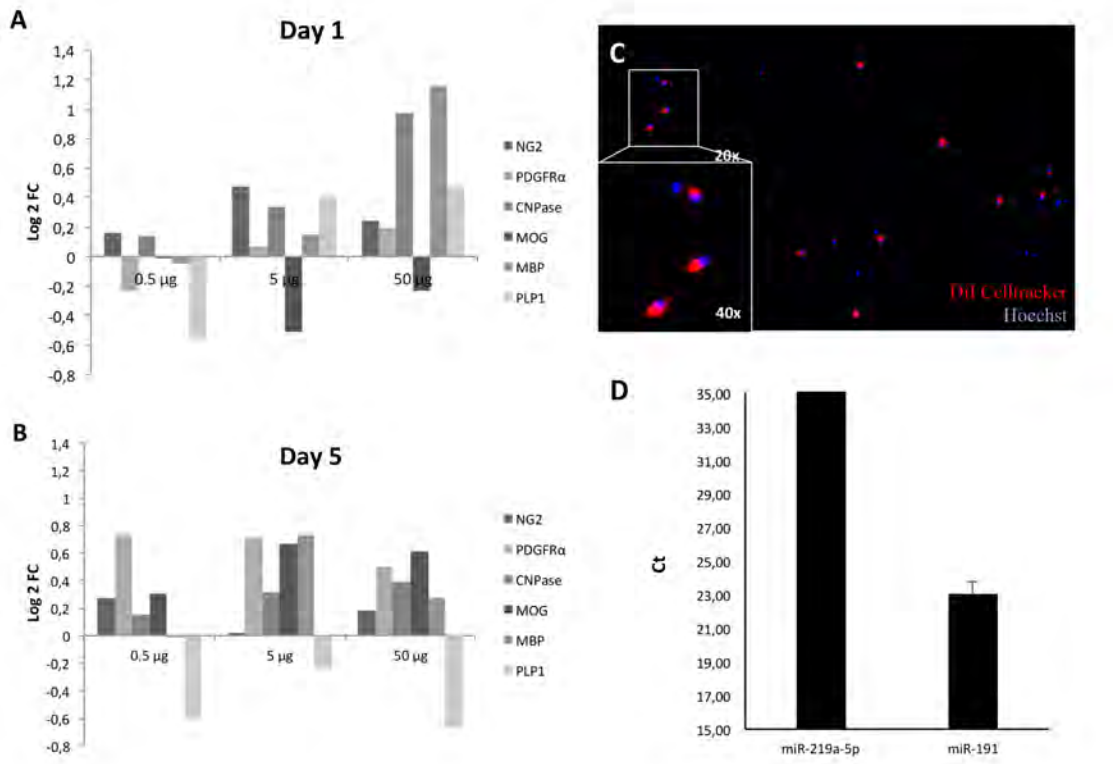

Figure 16: Exosomes do not promote OPC differentiation. A and B) qPCR of OPC culture at day 1 and 5 after exosome treatment is shown and compared with a non-treated culture, expressed as Log2 of the fold change. Genes of OPCs (NG2 and PDGFRα) of pre-oligodendrocyte (CNPase) and OLs (MOG, MBP and PLP1) do not show significant differences between groups (FC < 2). C) OPCs stained with Hoechst (blue) treated for 4 hours with Celltracker CM-DiI-labelled exosomes (red). Exosomes are taken up by OPCs with a 72 % efficiency. D) qPCR data of miR-219a-5p in young mice blood derived exosomes compared to the expression of miR-191 demonstrating that miR-219a-5p is not expressed in the exosomes, confirming data from arrays.

6. miR-219a-5p does promote OPC differentiation

To demonstrate that the lack of effect shown by our exosomes is due to the absence of miR-219a-5p in their cargo, miRIDIAN microRNA mmu-miR-219a-5p was administered to the OPC culture. Transfection efficiency was quantified using miRIDIAN microRNA Mimic Transfection Control with Dy547, which was 85% (*Figure 17, A*). Three days after the transfection with mmu-miR-219a-5p we found significant differences in gene expression pattern, showing an upregulation of myelin-related genes (MBP, PLP1, MOG and CNPase) and a downregulation of OPC-related

genes (NG2 and PDGFRα) when compared to miRIDIAN microRNA Mimic Negative Control treated OPCs (*Figure 17*, B). These results demonstrate that miR-219a-5p is able to promote OPC differentiation. Furthermore, immunofluorescence images of MBP and OLIG2 labelled OPCs culture show the presence of positive cells for MBP confirming that OPCs are differentiating into oligodendrocytes (*Figure 17*, C).

Next, to analyse if miR-219a-5p was able to promote or stimulate the remyelination process in a more complex model, miR-219a-5p was added to a lysolecithin demyelinated cerebellar organotypic culture. After 3 days in culture, qPCR was performed and an overexpression of OPCs related genes (NG2 and PDGFRα) and OLIG2 were detected. Myelin related genes were also up-regulated but not significantly (*Figure 17*, D).

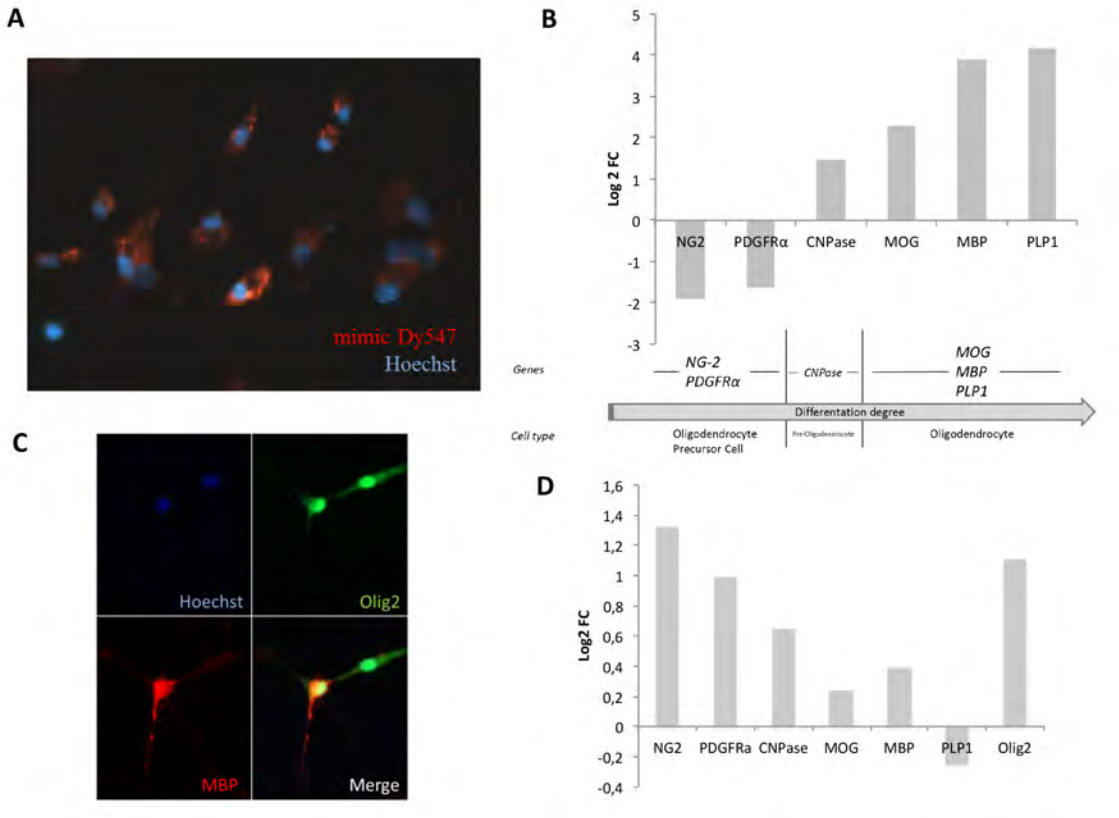

Figure 17: MiR-219 does promote OPC differentiation: A) Representative image of the transfection efficiency of mimic Dy547 in OPCs which is 85%. B) Gene expression patterns of OPC culture after treatment with miRIDIAN microRNA mmu-miR-219a-5p transfected with lipofectamine. MiRIDIAN microRNA Mimic Negative Control was used as a control. Results are shown as Log 2 of the fold change. C) Oligodendrocytes expressing MBP in an OPC culture demonstrating that miR-219 is able to promote the

expression of myelin related proteins D) Gene expression levels of the cerebellar organotypic culture treated with miRIDIAN microRNA mmu-miR-219a-5p after 3 days in culture.

DISCUSSION

Pusic et al. proposed that exosomes isolated from blood of young rats promote myelination in healthy and demyelinated hippocampal slice cultures of rats. Moreover, the intranasal administration of those exosomes in aged rats was proposed to increase myelin levels [182]. These interesting results opened a therapeutic way for MS patients for which no remyelination therapy is nowadays available. Nevertheless, the ability of pro-myelinating exosomes should also be addressed in a pathological animal model of MS. In the present work we studied the remyelination potential of young mice blood-derived exosomes in the EAE animal model under the hypothesis that young rodent blood-derived exosomes might improve clinical symptoms of the animals.

In our experiments, different concentrations of exosomes isolated from blood of young mice were tested, but no effect in the clinical score of the EAE model after intranasal administration was observed. Then, a cerebellar organotypic model of demyelination was used, in order to understand the effect of the young mice blood-derived exosomes in a less complex model. However, even if different exosome concentrations were tested, we were not able to find a pro-remyelinating effect in the treated samples. Our next step was to check these young mice blood-derived exosomes in OPCs culture with the aim of studying their cell differentiation potential. Again, no significant differences were found in OPC differentiation after the treatment with exosomes.

Pusic et al proposed that the pro-remyelination effect of the exosomes was related with their cargo, more specifically to pro-remyelinating microRNA in their cargos. MicroRNAs are single-stranded RNAs with around 22 base pairs that regulate gene expression. They have been shown to be involved in almost all biological processes (such as development, proliferation, differentiation and cell death) [260] and in several diseases like cancer or neurological diseases, and interestingly in MS [210,261–263]. One of the microRNAs proposed by these authors, the miR-219a-5p, has been described to take part in OPC differentiation, and to promote myelination by regulating the expression of genes related to the differentiation and/or myelination pathways [78,227,228].

With the aim of understanding the reason why young mice blood-derived exosomes were not promoting OPC differentiation, we decided to study the microRNA cargo with

a miRNA array. We detected more than 450 microRNAs in our exosomes, but surprisingly, miR-219a-5p was not present. This observation was confirmed by qPCR in two species (mice and rat exosomes). miR-138, miR-9, miR-23, miR-19b, miR-338 and miR-146a – microRNAs which have also been described to promote OPC differentiation [231,237,264] – were neither present in the cargo of the exosomes. We were able to detect miRNAs that has been described in this kind of samples as endogenous controls; so, we discard technical reasons for explaining the lack of miR-219a-5p in our exosomes. It has been widely described that the exosome concentration, cell of origin and cargo might be related to exogenous factors such as the recent ingestion of food, sport, diseases and environmental factors [265]. In this sense, it has also been proposed that environmentally enriched conditions might affect exosomes' content. Our data reinforce the hypothesis that the cargo of the exosomes is highly variable, which might be a limitation in their translations to clinical practice.

To confirm if miR-219a-5p was a key factor in the cargo of pro-remyelinating exosomes, we tested the ability of synthetic miR-219a-5p to promote OPC differentiation. We wanted to administer the microRNA directly into the cells, to make sure that the miRNA by its own was able to induce OPC differentiation. To do that, miRIDIAN microRNA mouse mmu-miR-219a-5p was administered with lipofectamine to OPCs, seeing an increase in the differentiation of OPCs to OLs. As miR-219a-5p was able to induce OPC differentiation by its own, miR-219a-5p could be an essential component of young mice blood-derived exosomes for the induction OPC differentiation and myelination. Moreover, as we have shown, when miR-219a-5p is not present in the cargo of exosomes, those are not able to induce the expected effect. In addition, these results are in concordance with recent reports that have shown that OPCs overexpressing miR-219a-5p had the ability to promote remyelination and functional recovery after their transplantation in the cuprizone-induced MS animal model [233]. MiR-219a-5p has also been shown to attenuate demyelination in the cuprizone model [234]. Moreover, Wang et al proved that miR-219a-5p was able to promote remyelination after lysolecithin induced demyelination [230]. Furthermore, it has been reported that human endometrial-derived stromal cells were able to be programmed into pre-oligodendrocytes by overexpressing miR-219a-5p [232].

Further, to confirm that miR-219a-5p was implicated in remyelination, we administered miRIDIAN microRNA mouse mmu-miR-219a-5p to a demyelinated cerebellar organotypic culture. For that we used again lipofectamine, a tested and proved method for the administration of microRNAs. We found an overexpression of OPC-related genes, such as NG2 and PDGFRα. Although the most accepted hypothesis is that miR-219a-5p promotes OPCs differentiation, there are new evidences suggesting that miR-219a-5p can also promote the expression of "precursor cell" genes, such as NG2 [233]. Interestingly, OLIG2 is also overexpressed, indicating an enrichment of the mature oligodendrocyte subpopulation. Besides, myelin related genes are also overexpressed, indicating that a possible enrichment in myelin is starting. Hence, as the mentioned results showed, miR-219a-5p could play a dual role in the remyelination process, promoting both the generation of new OPCs and their differentiation. Again, miR-219a-5p seems to be a key player in the promotion of remyelination and the lack of this microRNA in young mice blood-derived exosomes cargo can be a key factor in their remyelination failure.

In short, with this work we have reinforced the idea that the cargo of the exosomes influences their remyelination potential. More concretely, we conclude that miR-219a-5p is crucial for favoring remyelination and the enrichment of miR-219a-5p in the cargo of exosomes might be a therapeutic way to induce remyelination.

MATHERIAL AND METHODS

1. Exosome isolation and characterization

5-7 week-old C57BL/6 mice were anaesthetised with inhaled isoflurane 2% and sacrificed by cardiac puncture to obtain blood. A pool of 2-4 animals was used to obtain sufficient blood to extract enough exosomes to carry out the experiments. For this purpose, ExoQuick-TC (ExoQuick-TCxxA-1; System Biosciences) was used as previously described [151]. Briefly, blood samples were collected in citrate tubes, centrifuged at 1250 g for 15 minutes and the supernatant was recovered to obtain platelet-poor plasma (PPP). Then, 63 µl of ExoQuick-TC was added to 250 µl of PPP and incubated overnight at 4º C. Afterwards, to sediment the exosomes, two centrifugation steps were performed at 1500 g for 30 and 5 minutes, respectively. The pellet was resuspended in 200 µl of filtered DPBS.A Bradford assay was performed to quantify the protein levels of the samples, which were used to standardized exosome administration and for Western Blot (WB) analysis. Samples were frozen at -80º C until they were used.

In order to characterize the population of exosomes Nanoparticle Tracking Analysis (NTA) and WB were performed. NTA was performed in a NanoSight LM10 device (Malvern) as previously described [266]. Briefly, samples were diluted in filtered DPBS to get accurate acquisition (200-900 recorded tracks). Camera settings were fixed and maintained for all samples. For each sample, two videos of 1 minute were recorded and analysed with NanoSight NTA software 3.2 (Malvern) using the average count of the two duplicates for data representation

The expression of CD107a, CD71, and Grp78 were determined in EVs by WB as previously described [267]. Whole cellular extracts were used as controls in order to make sure that the exosome sample was pure and did not contain cellular debris. Briefly, 50µg of protein were denatured in sample buffer (50 mM Tris-Cl, 2% SDS, 10% glycerol and 0.1% bromophenol blue) by heat denaturation in three steps of 5 min at 37º C, 65º C and 95º C. Protein extracts were loaded in 10% sodium dodecyl sulphate polyacrylamide gel to performed the electrophoresis and electro-transferred to a nitrocellulose membrane (GE healthcare, USA). Membrane blocking was carried out by

adding 5% skim milk powder/tris-buffered saline (TBS)-5% tween (TBS-Tween) (Milk) after what membranes were incubated overnight at 4º C with anti-CD107a, anti-CD71, and anti-Grp78 (LS-C350569, Clone TiB-219 and G8918 Sigma, respectively) at 1:500 in blocking solution. After washing the membrane three times with TBS-0.1 % Tween-20 for 5 minutes, HRP-conjugated secondary antibody was used in blocking solution and incubated for 1 hour at room temperature (anti-rat IgG1 HRP 7077s 1:5000 for CD107a and CD71; anti-rabbit IgG1 7074s 1:40.000 for Grp78, both from Cell Signaling). For protein band visualization Luminanta^{TM} forte (Millipore) was used and images acquired using iBright FL1000 Imaging Systems (ThermoFisher).

2. EAE induction

All animal procedures were approved by the Biodonostia Health and Research Ethics Committee (CEEA17_002). In order to induce the model, 6-8 week-old female C57BL/6 mice (Charles River) were anaesthetised with isoflurane 2% and injected with 150 µg of myelin oligodendrocyte glycoprotein (MOG_{35-55}) (Ref: EPK1; Espikem), 1 mg of *Mycobacterium tuberculosis* (Ref:3114; Difco) and 100 µl of Freund's complete adjuvant (Ref:5881; Sigma) subcutaneously at two sites on the flanks at day 0. Mice were also intraperitoneally injected with 0.5 µg of Pertussis toxin (Ref: P2980; Sigma) at day 0 and 48 hours later. To measure the disease course, weight and clinical symptoms were observed and scored daily. To see the clinical scale, please refer to the supplementary information section, Supplementary Table 1 [268].

3. Organotypic culture

Newborn P7 C57BL/6 mice were sacrificed by decapitation, cerebellums were extracted and placed in organotypic culture medium, with BME 24 ml (Ref:41010; Thermo Fisher); 24% HBSS (Ref:24020091; Thermo Fisher); 24% Horse Serum (Ref:26050088; Thermo Fisher); 0.125% Glutamine (Ref: 25030024; Invitrogen); 1% antimycotic and antibiotic (Ref:A5955; Sigma); 3.5% Glucose (Ref: A1422; Panreac) for every 50 ml. 300-µm sagittal sections were taken using a McIlwain tissue chopper (McIlwain). Sections were separated and plated on a Millicell Cell Culture Insert membrane (Ref: PCIM ORG 50; Millipore) on a P6 plate and incubated in organotypic culture media at 37º C and 5% CO_2.

A demyelinating lesion was made as previously described [83]. Briefly, after 7 days in culture, media was removed and new media containing lysolecithin (0.5 mg/ml, Ref:L4129; Sigma) was added and incubated for 15 hours at 37º C and 5% CO_2. Then, lysolecithin media was removed and fresh media was added.

4. Oligodendrocyte precursor cell culture

The isolation of OPCs was done as previously described [59]. Briefly, postnatal P1-P3 C57BL/6 mice were sacrificed by decapitation. Once the brain was removed, meninges were extracted with the help of two forceps and a dissection microscope to obtain brain cortices. Then the tissue was digested mechanically and enzymatically with papain at 37º C for 14 minutes. 25 ml of OPC medium (DMEM (Ref:041966; Thermo Fisher), supplemented with 10% Foetal Bovine Serum (Ref: 16000044; Thermo Fisher) and 1% Antibiotic-Antimycotic (Ref:A5955; Sigma)) and 300 µl of a DNAse solution (1%, Ref:DN-25; Sigma) was added to stop papain digestion. The sample was centrifuged at 190 g for 10 min. The supernatant was removed and the pellet, after resuspension in 1 ml of OPC media, was filtered through a 100-µm nylon mesh strainer (Falcon). Finally, up to 8 ml of OPC media were added to the cells which were seeded in a 75-cm^2 Poly-L-Ornithine coated flask. After one week of incubation at 5% CO_2 and 37º C, renewing the media every 2-3 days, the culture was shaken for 15 hours at 200 rpm and one extra hour at 250 rpm. After that, the media was recollected and passed through a 40-µm nylon cell strainer (Falcon) and centrifuged at 190 g for 10 minutes. The pellet was resuspended in 10 ml of OPC media. The suspension was plated on an untreated plastic Petri dish at 37 ºC for one hour to allow the microglial cells to attach to the Petri dish. Then, the media was recollected, centrifuged at 190 g for 10 minutes, filtered again through a 40-µm strainer and incubated in a new uncoated Petri dish for 30 minutes. After the 30 minutes, the media was recollected, and centrifuged at 190 g for 10 minutes to finally obtain the purified OPCs. Cells were incubated in a laminin-treated plate to perform the experiments.

5. Exosome administration

In EAE: to allow a direct and feasible route of administration facilitating exosomes to reach the CNS, those were intranasally administered as previously described. Briefly

once the animals were anaesthetised with inhalatory anaesthesia with isoflurane 2%, four administrations of 5 µl each were given alternating nostrils. To find out if the exosomes had an immunomodulatory or remyelination potential, exosomes were administered at two time points in several experiments. Firstly, to test the remyelination capacity of the exosomes and assess whether they were able to reduce the clinical score once the symptoms appeared, exosomes were administered at the disease onset (score = 1), at two different concentrations (DPBS as vehicle, 100 µg of quantified protein; replicating the data published by Pusic et al; and a higher concentration of 200 µg of quantified protein) (n = 6 per group). To analyse the immunomodulatory potential of exosomes and evaluate if they were able to cause a delay in the disease onset, they were administered 4 days after EAE induction (vehicle and 100 µg of quantified protein), (n = 3 and 4 respectively).

In organotypic culture: 10µl of exosomes at different concentrations (10 µg, 20 µg, 40 µg, 80 µg, 160 µg and 240 µg of quantified protein) diluted in DPBS were administered to each cerebellum slice (in triplicates) after replacing lysolecithin with fresh media and cultured for three days. Then confocal microscopy analysis was performed.

In OPCs: 24 hours after cells were plated, exosomes were added at 0.5, 5, 50 and 100 µg of quantified protein and maintained in culture for one and five days prior to RNA isolation. Triplicates were carried out. To confirm that exosomes were taken up by OPCs, exosomes were labelled with Dil (Celltracker CM-DiI, Thermo Fisher) as previously described [181]. Briefly, exosomes were incubated with 1µg/ml of Celltracker CM DiI for 5 minutes at 37º C and 15 minutes at 4º C. Then the sample was centrifuged at 20.000 g for 20 minutes in order to pellet exosomes and remove excess of dye. Then, exosomes were resuspended in 10 µl of DPBS and added to the OPCs culture. After four hours, cells were fixed, labelled with Hoechts and images were taken with 20X magnification in a Nikon Eclipse 80i (Nikonn) using NIS elements AR 3.2 (Nikon). Fields were selected in an aleatory manner. Positive and negative cells for Celltracker CM DiI were count.

6. MiR-219a-5p transfection

For miR-219a-5p transient overexpression, miRIDIAN microRNA mmu-miR-219a-5p and miRIDIAN microRNA Mimic Negative Control were used, both purchased from Dharmacon. miRIDIAN microRNA Mimic Transfection Control with Dy547 (Dharmacon) was used as a control of the transfection efficiency. miRIDIAN microRNA transfection was carried out using Lipofectamine RNAiMAX (Ref: 13778030; Thermo Fisher) following the manufacturer's protocol.

In OPCs: 24 hours after culturing the OPCs, 25 nM of miRIDIAN microRNA mimics were incubated in 50 µl of Opti-MEM medium (Ref:31985062; Thermo Fisher) containing 1,25 µl of Lipofectamin reagent for 5 minutes. After this period, the mix was added to each well containing 450 µl of OPC medium. After 24 hours, cells were fixed and labelled with Hoechts to quantify the percentage of transfected cells with miRIDIAN microRNA Mimic Transfection Control with Dy547. For functional analysis, three days after transfection, RNA was isolated. No media changes were done during this time.

In cerebellar organotypic culture: after removing lysolecithin 25nM of miR-219a-5p mimic per slice were incubated in 50 µl of Opti-MEM medium containing 1,25 µl of Lipofectamine reagent for 5 minutes. Three days after transfection, RNA was isolated. No media changes were done during this periods.

7. Immunofluorescence and confocal microscopy

Three days after the administration of the exosomes, cerebellar slices were fixed with 4% paraformaldehyde for 40 minutes at 25º C. Then, the slices were washed with DPBS and blocked in blocking solution (0.5% Triton (Ref: T8787; Sigma) and 10% goat serum (Ref: G9023; Sigma) in DPBS) for one hour. Primary antibodies against myelin basic protein (MBP) (Ref: Ab7349; Abcam) and neurofilament (NFL) (Ref: Ab8135; Abcam) (both 1:200 in block solution) were added and incubated overnight at 4º C. The next day, samples were washed with 0.1% Triton in DPBS and incubated with secondary antibodies for two hours in blocking solution. Secondary antibodies conjugated with Alexa Fluor 594 nm for MBP and Alexa Fluor 488 nm for NFL were used (Ref: A21209 and 21206; Thermo Fisher). Then three washes were done with

0.1% Triton in DPBS and stained with Hoechst (Ref: B2261; Sigma) 10% in DPBS for 10 minutes. After two extra washes with DPBS samples were mounted in Fluoromount-G (Ref: 0100-01; SouthernBiotech). Stacks of images were acquired by confocal microscopy using an LSM 510-Meta (Zeiss). Axonal tracks were sampled acquiring at least three images per slice and three slices per condition making a total of 9 images per condition. Images were obtained at 1μm intervals in white matter areas at x63 (1.4 NA) magnification for both channels sequentially, to avoid crosstalk. To conduct the analysis of the remyelination degree, maximum intensity projection of 10 intervals and Colocalization Threshold plugin from open source software ImageJ were used to calculate Manders Colocalization Coefficient, where 0 represents non-colocalization and 1 perfect spatial colocalization [269].

To carry out the immunofluorescence to detect the expression of myelin related proteins in OPC culture, three days after miRIDIAN microRNAs were added, OPCs were fixed with 4% paraformaldehyde for 5 minutes at 25º C. Then, cells were washed with DPBS and blocked in blocking solution (0.5% Triton (Ref: T8787; Sigma) and 10% goat serum (Ref: G9023; Sigma) in DPBS) for one hour. Primary antibodies against myelin basic protein (MBP) (Ref: Ab7349; Abcam) and oligodendrocyte transcription factor 2 (OLIG2) (Ref: Ab9610; Chemicon International) (both 1:200 in block solution) were added and incubated overnight at 4º C. The next day, samples were washed with 0.1% Triton in DPBS and incubated with secondary antibodies for two hours in block solution. Secondary antibodies conjugated with Alexa Fluor 594 nm for MBP and Alexa Fluor 488 nm for OLIG2 were used (Ref: A21209 and 21206; Thermo Fisher). Afterwards three washes were done with 0.1% Triton in DPBS and stained with Hoechst (Ref: B2261; Sigma) 0.1% in DPBS for 5 minutes. After two more washes with DPBS samples were mounted in Fluoromount-G (Ref: 0100-01; SouthernBiotech). Images were acquired in a Nikon Eclipse 80i (Nikonn) and analysed using NIS elements AR 3.2 (Nikon).

8. RNA isolation

From OPCs: One and five days after exosomes were administrated, total RNA was isolated from OPCs using the miRNeasy Mini Kit (Ref: 217004; Qiagen) following manufacturer's instructions. Three days after OPCs were treated with miRIDIAN

microRNAs total RNA was isolated as previously described. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer.

From Cerebellar Organotypic Culture: Three days after transfection with miRIDIAN microRNAs the membrane where the slices were grown up was cut out, 700 µl of Qiazol were added and vortexed for one minute. Then total RNA was isolated form cerebellar organotypic culture using the miRNeasy Mini Kit (Ref: 217004; Qiagen) following manufacturer's instructions. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer.

From Exosomes: RNA isolation was done as previously described [151]. miRNeasy Mini kit (Qiagen) was used to extract RNA from 500 µl of exosome samples. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer.

9. Quantitative PCR

For microRNA 219: RNA was reverse transcribed using TaqMan™ MicroRNA Reverse Transcription Kit (Ref: 4366596; Thermo Fisher) following manufacturer's protocol in a Veriti Thermal Cycler (Applied Biosystems). miR-219a-5p expression analysis was performed using TaqMan™ Universal Master Mix II (Ref: 440043; Thermo Fisher) and miRNA TaqMan Assay (ID:522; Thermo Fisher). The qPCR was carried out in a CFX384 Thermal Cycler (Bio-Rad). Raw data was processed in Bio-Rad CFX Manager software.

For genes: Total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Ref: 4368814; Thermo Fisher) following manufacturer's protocol in a Veriti Thermal Cycler (Applied Biosystems). Gene expression analysis for OPC differentiation was performed in order to establish the differentiation degree of the cells, both in the OPC culture and in the organotypic culture, using KiCqStart SYBR Green qPCR ReadyMix (Ref:KCQS02; Sigma) and KiCqStart SYBR Green Primers (Ref: KSPQ12012; Sigma). The expression levels of the following genes were analysed: platelet-derived growth factor α (PDGFRα) and neuron-glial antigen 2 (NG2) to determine OPCs; 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase) to determine pre-oligodendrocytes; myelin oligodendrocyte glycoprotein (MOG), myelin basic

protein (MBP), proteolipid protein (PLP1) to determine oligodendrocytes; ribosomal protein L13α (RPL13α) and phosphoglycerate kinase 1 (PGK1) were used as endogenous controls. The qPCR was carried out in a CFX384 Thermal Cycler (Bio-Rad). Raw data was processed in Bio-Rad CFX Manager software and the subsequent analysis to calculate relative expression was carried out in Excel software using the 2^{-DDCT} method [270].

10. MicroRNA microarray

Total RNA (500 ng) was labelled using the FlashTag Biotin labelling kit (Ref: 901911;Thermo Fisher) and hybridised to the GeneChip miRNA 4.0 Array (Ref: 902412; thermo Fisher), which covers 1908 mouse miRNAs, following the manufacturer's instructions. Briefly, RNA molecules were polyadenylated and a biotin-labelled DNA molecule was attached in a subsequent ligation step. Finally, labelled RNA was hybridised to the array, washed and stained in a GeneChip Fluidics Station 450 and scanned in a GeneChip Scanner 7G (Affymetrix).

11. Statistical analysis

For the EAE animal model, the area under the curve (AUC) of the overall disease severity was calculated for each mouse to analyse the disease severity for each group using non-parametric statistical tests (Wilcoxon rank-sum test) [271]. Arrays were normalised by RMA using Expression Console software from Affymetrix.

CHAPTER TWO: Development of a new EAE clinical score and a mobile application to monitor the model.

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(Submitted)

CHAPTER THREE: MiR-219a-5p characterization in relapsing-remitting multiple sclerosis patients and healthy controls

INTRODUCTION

Myelin is a lipid membrane that wraps axons, giving them trophic support and allowing the transmission of nerve impulses [10]. However, in several diseases, such as multiple sclerosis (MS), this myelin is damaged by an autoimmune attack. In brief, activated T cells cross the blood brain barrier and damage oligodendrocytes; the cells responsible for generating myelin in the central nervous system (CNS). This leaves axons unprotected and blocks the saltatory nerve impulse transmission. Fortunately, after a demyelinating insult the body is able to regenerate the lost myelin in a process called remyelination. To do this, oligodendrocyte precursor cells need to proliferate, migrate, differentiate to oligodendrocytes and generate new myelin [21]. After remyelination, saltatory nerve impulse transmission is recovered. This form of the disease, in which a period of neurological symptoms (relapse) is followed by a recovery stage (remission), is classified as relapsing-remitting MS (RRMS). This is the most common sort of MS, representing approximately 85% of patients [279]. However, after several cycles of demyelination and remyelination this process tends to fail, leading the patient to a progressive form of the disease. Unfortunately, nowadays there is no treatment available to induce the regeneration of the myelin and the scientific community is elucidating new strategies to stimulate myelin regeneration and therefore neuroprotection.

With regard to this, microRNAs have appeared as promising candidates to induce remyelination. They are small single-stranded RNAs (20-22bp) with regulatory functions by targeting sequence-specific messenger RNAs. One of these candidate microRNAs is miR-219a-5p, which has been shown to be a key factor in OPC differentiation and therefore in remyelination [230,231]. There are several works in mice models and transgenic animals that reinforce the role of miR-219a-5p in remyelination [228,229,233]. Interestingly, when a synthetic miR-219 was administered intrathecally to the experimental autoimmune encephalomyelitis animal model, a decrease in the clinical score was shown due to the generation of new myelin forming oligodendrocytes [230]. In addition, as shown in Chapter One, miR-219a-5p might be a key factor in the cargo of exosomes to induce remyelination.

All this evidence suggests that miR-219a-5p could also be playing a role in human remyelination and therefore, we hypothesized that expression differences could be detectable between relapse and remission in MS patients.

In this chapter, the expression of miR-219a-5p in blood patients in relapse and remission together with healthy controls will be analysed in order to understand better the role that this microRNA could be playing in remyelination.

MATERIAL AND METHODS

Blood sample collection

Whole blood (10 ml) was collected from 10 patients with RRMS and 10 healthy donors in the Department of Neurology at Donostia University Hospital. Two samples from each MS patient were collected: one during a relapse and another during remission. A relapse was defined as an episode of new neurological symptoms of at least 24-h duration, not associated with fever or infection [280]. Relapse blood samples were collected before giving any corticosteroid treatment. Healthy controls (HC) were matched for age and sex. The same blood sample was used to isolate RNA from leucocytes, plasma and EVs. Samples from all donors were collected after receiving written informed consent. The study was approved by the hospital's ethics committee and samples have been processed and stored at the Basque Biobank (www.biobancovasco.org).

RNA isolation from leucocytes

Total RNA was isolated from peripheral blood leucocytes with the LeukoLOCK kit (Ambion) using the alternative protocol to capture small RNAs. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer and RNA integrity was assessed using a bioanalyzer with the RNA 6000 Nano Assay Protocol (Agilent Technologies). Only samples with a RNA integrity number higher than 6 were included. *This protocol was carried out in the context of a previous article by our group* [261].

MicroRNA microarray hybridization

Total RNA (500 ng) was labeled using the FlashTag Biotin labelling kit (Genisphere) and hybridized to the GeneChip miRNA 1.0 Array (Affymetrix), which covers 847 and 922 human miRNAs and snoRNAs, respectively, following the manufacturer's instructions. Briefly, RNA molecules were polyadenylated and, in a subsequent ligation step, a biotin-labelled DNA mole- cule was attached. Finally, labelled RNA was hybridized to the array, washed and stained in a GeneChip Fluidics Station 450 and scanned in a GeneChip Scanner 7G (Affymetrix). *This experiment was carried out in the context of a previous article by our group* [261].

Microarray data analysis

Raw data analysis was first performed, including a detection step (a probe set is detected above background with an associated p-value) resulting in a true/false call and a quantile normalization step using the miRNA QC Tool software (Affymetrix).

RNA isolation from Plasma and Extracellular Vesicles.

Plasma was obtained after centrifuging blood samples at 1500g for 15 minutes. Plasma samples were then used to obtain extracellular vesicles following a differential centrifugation step protocol as previously described [151]. Trizol LS (#10296028, Thermo-Fisher) was used to isolate RNA from both plasma and extracellular vesicle samples following manufacturers´ protocol. RNA was quantified by NanoDrop ND-1000 spectrophotometer.

miR-219a-5p quantification by ddPCR

RNA was reverse transcribed using TaqMan™ MicroRNA Reverse Transcription Kit (# 4366596; Thermo Fisher) following manufacturer's protocol in a Veriti Thermal Cycler (Applied Biosystems). MiR-219a-5p expression analysis was performed using miRNA TaqMan assay (ID:522; Thermo Fisher) and QX200 droplet digital PCR system (BioRad). Analysis was carried out using QuantaSoft 1.6.6 software (BioRad) and data was expressed as the number of copies of miR-219a-5p per nanogram (ng) of total RNA. Non-parametric analysis was carried out by using Kruskal-Wallis test.

RESULTS AND DISCUSSION

After a relapse, patients are able to regenerate the lost myelin. MiR-219a-5p has shown to positively mediate OPCs differentiation and remyelination. With the aim of understanding if the microRNA could be playing a role in patients remyelination after a relapse, we analysed miR-219a-5p levels in both relapse and remission, and also HC, expecting to find higher levels after relapse, as an indicator of remyelination. In the context of a previous work published by our group in which global microRNA expression pattern in leucocytes of relapse and remission patients were analysed by microarray [261], we checked this data to specifically determine miR-219a-5p levels in the same patients. As expected, microarray did not detect miR-219a-5p in relapse, remission nor HC samples. However, miR-219a-5p expression is expected to occur in the CNS and not in leucocytes, which are not involved in myelin regeneration. With the hypothesis that microRNAs that are generated in the CNS can be released into the blood, we decided to analyse plasma and extracellular vesicles (EVs) miR-219a-5p levels of the same patients and HC and compare them to leucocytes expression levels. In order to compare these samples, we decided to use droplet digital PCR to quantify with precision and high sensitivity the number of copies of miR-219a-5p per nanogram of total RNA.

Interestingly, we were able to detect miR-219a-5p in the three types of samples. Although miR-219a-5p level in leucocytes was closed to cero, these results were of high interest as microarray was not able to detect miR-219a-5p expression in in the same sample, indicating that ddPCR is more sensitive than microarrays. Moreover, we compared miR-219a-5p expression levels in the three samples determining that EVs contained more copies of miR-219a-5p per nanogram of total RNA when compared to leucocytes or plasma ($p<0,001$ for both comparisons) (Figure 19, A). EVs are cell-released vesicles that contain proteins and genetic material and that have been involved in cellular communication. In addition, EVs have been shown to contain a higher and different proportion of microRNAs than their parent cells [281], indicating that could be a method to concentrate microRNAs. Interestingly, CNS derived-EVs have been proposed to cross the blood brain barrier and to be found in blood [282]. In addition, miR-219a-5p has been described to be highly expressed in the CNS when compared to other

tissues (GTEx). These evidences made us think that a great proportion of EVs-derived miR-219a-5p could be derived from the CNS.

Furthermore, we wanted to analyse if miR-219a-5p could be increased in relapse when compared to remission and healthy controls. When we made this comparison in leucocytes, no significant differences were found (Figure 19, B). Obviously, and as explained before, leucocytes are not involved in the myelin regeneration and no differences in the levels of miR-219a-5p expression were expected. Plasma samples showed an interesting pattern (with no significant differences), in which miR-219a-5p copies per nanogram of RNA were higher in relapse samples when compared to remission ones and the latter higher than in HC (Figure 19, C). However, due to the low miR-219a-5p levels in both leucocytes and plasma samples we consider that EVs could be the most representative sample to be analysed, as miR-219a-5p was found to be enriched in EVs. When we analysed the expression of miR-219a-5p in EVs, we detected higher levels in relapse versus remission, which could highlights the role that miR-219a-5p is playing in active remyelination. Interestingly, relapse levels were similar to HC (Figure 19, D). These results could indicate that during remission, expression of miR-219a-5p is decreased as a consequence of a depletion in the remyelination machinery but after a relapse, this machinery makes an effort in order to compensate the lost myelin reaching HC miR-219a-5p levels. However, these results did not show significant differences between groups, probably because of the low number of samples and a poor statistical power, and we should be careful when interpreting these results.

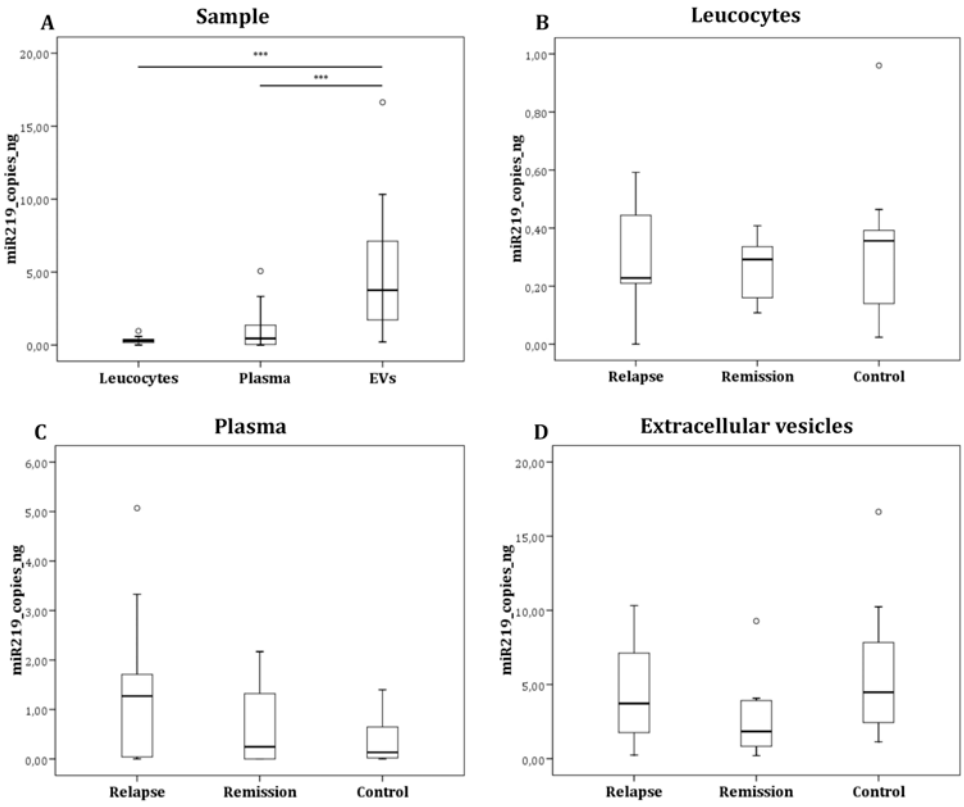

Figure 19: miR-219a-5p copies per nanogram of RNA in Leucocytes, plasma and EVs (A). Relapse, remission and HC miR-219a-5p copies per nanogram of RNA in leucocytes (B), plasma (C) and EVs (D).

CONCLUSIONS

We have shown that the isolation of EVs increases the microRNA concentration, making them a potential source for microRNA studies. In addition, EVs isolated from blood are an easy and feasible sample to work with in order to understand molecular pathways that are involved in CNS diseases. Finally, these results indicate that miR-219a-5p might mediate in the remyelination process that takes place in MS patients. However, further experiments are needed in order to increase sample population and to obtain more robust results.

CHAPTER FOUR: MiR-219a-5p enriched exosomes induce OPC differentiation and EAE improvement more efficiently than liposomes and nanoparticles. A comparative study.

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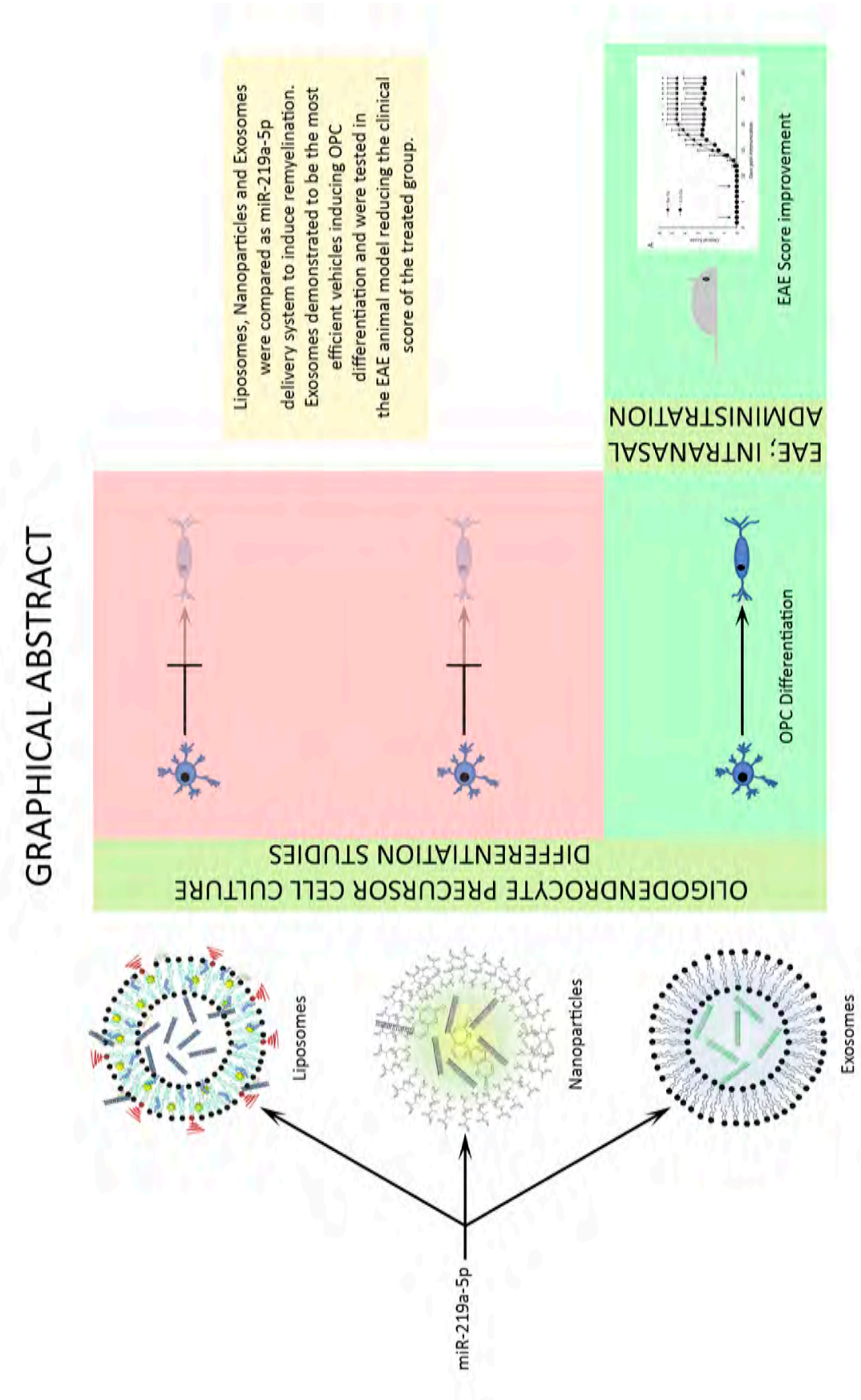


Figure 20: Graphical abstract

INTRODUCTION

Myelin is a lipid membrane formed by oligodendrocytes in the central nervous system (CNS). Myelin wraps axons giving them trophic support and allowing a correct transmission of nerve impulses. However, in several diseases this myelin is damaged causing an incorrect nerve impulse transmission and an imbalance in the homeostasis of axons, which can lead to neurodegeneration. The most common demyelinating disease is multiple sclerosis (MS), a chronic CNS disease in which myelin is damaged by an autoimmune attack. In brief, activated T cells migrate across the blood brain barrier (BBB), and induce an autoimmune response against the myelin [273]. Interestingly, in the first stages of the disease, myelin can be endogenously restored in a process called remyelination. To do this, oligodendrocyte precursor cells (OPC) proliferate, migrate to the lesions, differentiate to oligodendrocytes and finally extend myelin's sheaths around axons [21].

Nevertheless, when the disease progresses the remyelination potential decreases and tends to fail. The reasons why this occurs are still not clear but it is thought to be related with a lack of OPC, and insufficient migration or a poor differentiation [41,283].

Nowadays there is a wide rage of treatments that are focused on the attenuation of the immune response but there is still no treatment that promotes the regeneration of the myelin and therefore alternative strategies are appearing. The detection of undifferentiated Ol in demyelinating lesions suggests that the stimulation of the differentiation process might be a feasible and potential way of inducing remyelination in MS patients [47].

With regard to this, several microRNAs have appeared as OPC differentiation mediators. MicroRNAs are small non-coding RNA formed by about 22 base pairs with regulatory functions. More concretely, miR-219a-5p has been widely used in experimental analysis demonstrating that it is able to generate OPC differentiation and therefore remyelination [182,228,230,231,233,284], as we also showed in Chapter One. However, the administration of microRNAs to the CNS is a tricky question and only invasive administration methods have been tested in animal models [230,234]. Although these experiments showed positive results, direct administration of microRNA to the CNS is

not feasible as a treatment for MS patients. In order to administer the treatment in a non-invasive and efficient way, several delivery systems have been developed. The idea behind them is to dispense the microRNA to the CNS non-invasively and in a controlled manner, inducing OPC differentiation and remyelination.

In this work we have quantified and compared the ability of two synthetic systems (nanoparticles and liposomes) and one biological (exosomes), as microRNA carriers and their ability to induce OPC differentiation in a primary oligodendrocyte precursor cell culture. In addition, the Experimental Autoimmune Encephalomyelitis model has been used to study the remyelination potential of the most promising delivery system.

METHODS

microRNA carriers generation and loading (Figure 21).

Liposomes

Mimic mmu-miR-219a-5p (mimic-219a-5p) and mimic Transfection Control with Dy547 (mimic-Red) (#C300576 and #CP004500 Dharmacon) enriched liposomes or empty liposomes were prepared by the lipid film rehydration method [285,286]. Mixtures of lipids (typically 10 μmol) were prepared in a 6:1 v/v mixture of chloroform: methanol. The final mixtures contained 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (molar fraction: x=0.167), 1,2-dioctadecanoyl-sn-glycero- 3-phosphocholine (DSPC: x=0.617) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethyleneglyco l)-2000] (ammonium salt) (PEG-DSPE: x=0.05) (Avanti Polar Lipids (AL, USA)). Some of the liposome formulations were fluorescently labelled by adding 13μL of 3,3′-Dioctadecyloxacarbocyanine perchlorate dissolved in chloroform (1mg/mL) (DOiC) to the lipid mixture before the lipid film formation. Lipid films were formed by evaporation of chloroform: methanol on a rotavapor operated under vacuum at 30°C and 2 hours drying under a nitrogen flow. Lipid films were hydrated with 2 mL of RNAse-free water at 65°C. MiRNAs were added in a ratio of miRNA:lipid of 0.9 nmol miRNA:μmol lipid. The lipid film with the miRNA was allowed to hydrate overnight at 4 ºC forming miRNA-PEgylated liposome complexes. After that, the liposome dispersions were diluted twice with water and extruded 16 times at 65 ºC through polycarbonate membrane filters (Whatman, Rentfort, UK) of decreasing pore diameter (400, 200 and 80 nm). After this process, miR-219a-5p-liposomes (219-Lp), mimic-Dye-547-liposomes (Red-Lp) and empty liposomes (E-Lp) were obtained. This protocol was carried out in CICbiomaGUNE.

Nanoparticles

Poly lactic-co-glycolic acid (PLGA) nanoparticles were synthesized using water in oil in water (w/o/w) double emulsion technique. In brief, miRIDIAN microRNA mmu-miR-219a-5p or miRIDIAN microRNA Mimic Transfection Control with Dy547 were dissolved in 200 μl of surfactant (0.5% poly vinyl alcohol (PVA) as an excipient), and were homogenized with 10% w/v PLGA in dichloromethane with a Misonix sonicator probe (Misonix, Inc., NY, USA) at 10W for 3 min. This initial water in oil emulsion (w/o) was then added to 15 ml of 5% w/v solution of PVA for a second emulsion step

and was homogenized at 12W for 3 min. The resultant water in oil in water (w/o/w) double emulsion was then subjected to organic solvent evaporation under stirring at 600 rpm overnight at room temperature. The sample was centrifuged in water, 4 times at 14.000 rpm, to wash out any water-soluble surfactant and polymer residues. Finally, miR-219a-5p-nanoparticles (219-Np), mimic-Dye547-nanoparticles (Red-Np) and empty nanoparticles were obtained (E-Np). This protocol was carried out in TECNALIA.

Exosomes

pLKO.1 lentiviral particles containing miR-219a-5p sequence or empty particles were infected in HEK293T cells. Selection was made by culturing the cells with puromycin (Cayman Chemical) and cells were maintained with this antibiotic for the rest of the experiment to avoid the growth of non-infected cells. To isolate exosomes, cells were grown to a 80% confluence in media (DMEM Thermo Fisher, 10% FBS Thermo Fisher and 1% Puromycin) followed by a change in the media to OptiMEM (Thermo Fisher) containing puromicyn for 36 hours. Then, media was recollected and exosomes concentrated using Tangential Flow Filtration system with VivaFlow 50R 10.000MW membrane (Sartorious) followed by the use of centrifugal filters of 10.000MW (Merck Millipore). Afterwards, exosomes were isolated by differential centrifugation steps. Briefly, the sample was centrifuged at 13.000g for two minutes, supernatant recollected and centrifuged again at 20.000g for 20 minutes. Then pellet was recollected and resuspended in dPBS. With this process miR-219a-5p enriched exosomes (219-Ex) and non-enriched exosomes (Ne-Ex) were obtained. A Bradford assay was performed to quantify the protein levels of the samples, which was used to standardise exosome administration.

In order to obtain fluorescent exosomes for up-take experiments exosomes were labelled with CM-Dil (Celltracker CM-DiI, #C7001, Thermo Fisher) as previously described [287]. In short, exosomes were incubated with 1 µg/ml of Celltracker CM DiI for 5 minutes at 37º C and 15 minutes at 4º C. Then the sample was centrifuged at 20.000 g for 20 minutes in order to pellet exosomes and remove the excess of dye. Exosomes were resuspended in dPBS.

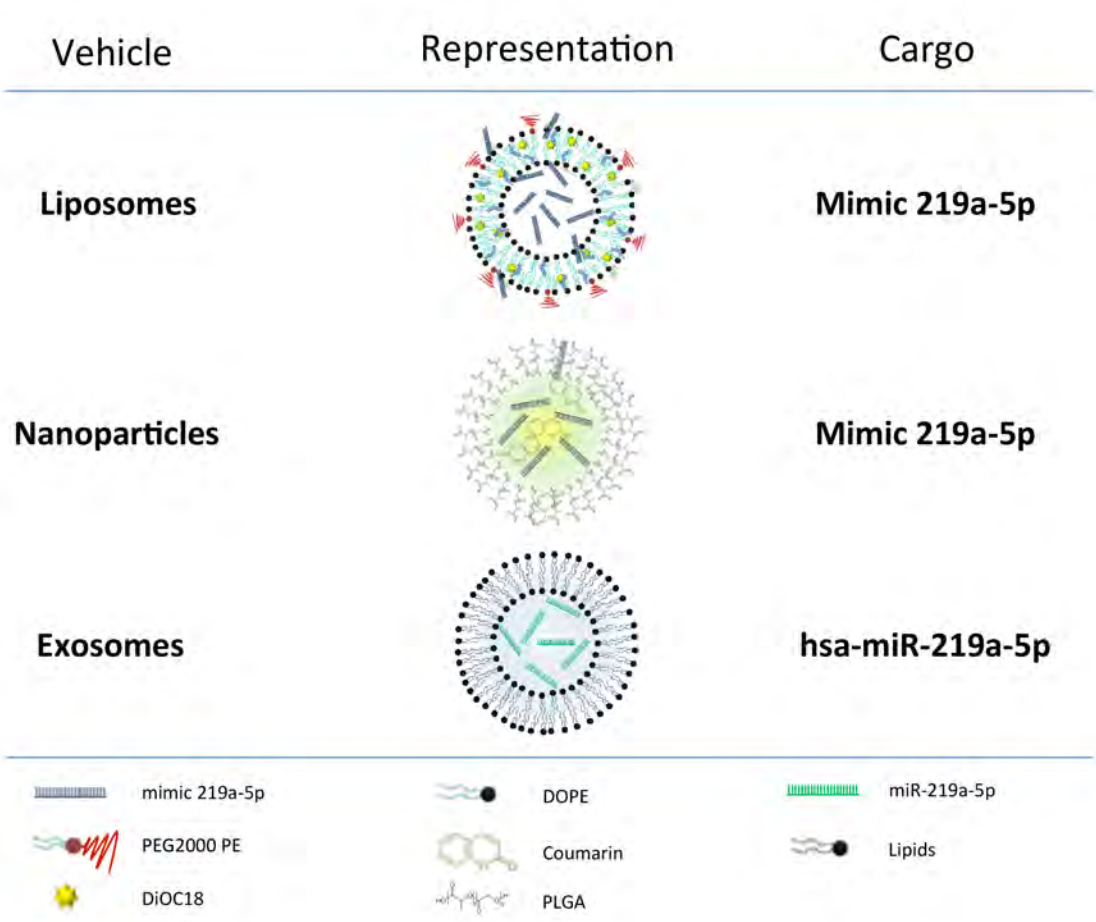


Figure 21: Schematic representation of liposomes, nanoparticles and exosomes with their main components and the type of miR219a-5p they were loaded with. Liposomes and nanoparticles were loaded with synthetic microRNA. In contrast, exosomes contained biologically produced microRNA.

Characterization of microRNA carriers

Nano Tracking Particle Analysis (NTA)

NTA was performed in a NanoSight LM10 device (Malvern) as previously described [266]. In brief, samples were diluted in filtered dPBS to get accurate acquisition (200-900 recorded tracks). Camera settings were fixed and maintained for all samples (gain: 6.9; camera level: 5). For each sample, two videos of 1 minute were recorded and analysed with NanoSight NTA software 3.2 (Malvern). Total particles counts were obtained and profile distribution graphs generated.

Droplet Digital PCR

RNA encapsulated in liposomes, nanoparticles and exosomes was isolated using Trizol LS protocol following the manufacturer´s instructions (#10296028, ThermoFisher). Exosomes RNA was quantified by NanoDrop ND-1000 spectrophotometer. Levels of RNA in liposomes and nanoparticles were not quantified by NanoDrop ND-1000 due to the low yields of total RNA in the sample (only mimic-219a-5p was present). RNA was reverse transcribed using TaqMan™ MicroRNA Reverse Transcription Kit (# 4366596; Thermo Fisher) following the manufacturer's protocol in a Veriti Thermal Cycler (Applied Biosystems). miR-219a-5p expression analysis was performed using miRNA TaqMan Assay (ID: 522, Thermo Fisher) and supermix for probes (#186-3023, BioRad) in a QX200 droplet digital PCR system (BioRad). Analysis was carried out using QuantaSoft 1.6.6 software (BioRad). In order to compare results between vehicles, the number of copies of miR-219a-5p were related to the number of particles quantified by NTA.

CryoTEM

Liposomal, nanoparticle and exososomal solutions were vitrified following standard protocols described elsewhere [288]. Quantifoil holey carbon film grids (Orthogonal Array of 2µm Diameter Holes - 2µm Separation, mounted on a 300M Cu grid, #657-300-CU, Ted Pella) were vitrified in liquid ethane in Vitrobot (FEI) after a negative glow-discharged treatment of the grids and deposition of 3 µL of each sample. Cryo-transfer sample holders of type GATAN Model 626 kept the sample vitrified during electron microscopy analysis. Every sample was observed on a JEM-2100F UHR (80-200kV, JEOL, Ltd.) field emission gun (FEG) transmission electron microscope at different magnifications (8000x and 30 000x). Low-dose micrographs were recorded on a state of the art TVIPS F216 CMOS camera (2k x 2k). This protocol was carried out in CICbiomaGUNE.

MicroRNA microarray

Following manufacturer's instructions, total RNA (500 ng) was labelled using the FlashTag Biotin labelling kit (Ref: 901911;Thermo Fisher) and hybridised to the GeneChip miRNA 4.0 Array (Ref: 902412; Thermo Fisher), which covers 1908 mouse miRNAs. Briefly, RNA molecules were polyadenylated and a biotin-labelled DNA

molecule was attached in a subsequent ligation step. Finally, labelled RNA was hybridised to the array, washed and stained in a GeneChip Fluidics Station 450 and scanned in a GeneChip Scanner 7G (Affymetrix).

Oligodendrocyte precursor cell culture

The isolation of OPCs was carried out as previously described [59]. Briefly, postnatal P1-P3 C57BL/6 mice were sacrificed by decapitation. Once the brain was removed, meninges were extracted and tissue digested mechanically and enzymatically with papain at 37ºC for 14 minutes. 25 ml of OPC medium (DMEM (#041966; Thermo Fisher), supplemented with 10% Foetal Bovine Serum (# 16000044; Thermo Fisher) and 1% Antibiotic-Antimycotic (#A5955; Sigma) was added to the sample. Then, 300 µl of a DNAse solution (1%, #DN-25; Sigma) was added to stop papain digestion. After a centrifugation step at 190 g for 10 min, supernatant was removed and the pellet, after resuspension in 1 ml of OPC media, was filtered through a 100-µm nylon mesh strainer (Falcon). Finally, up to 8 ml of OPC media were added to the cells which were seeded in a 75-cm^2 Poly-L-Ornithine coated flask. After one week of incubation at 5% CO_2 and 37ºC, renewing the media every 2-3 days, the culture was shaken for 15 hours at 200 rpm and one extra hour at 250 rpm. This was followed by media collection, which was passed through a 40-µm nylon cell strainer (Falcon) and centrifuged at 190 g for 10 minutes. The pellet was resuspended in 10 ml of OPC media. The suspension was plated on an untreated plastic Petri dish at 37 ºC for one hour to allow the microglial cells to attach to the Petri dish. Then, the media was collected, centrifuged at 190 g for 10 minutes, filtered again through a 40-µm strainer and incubated in a new uncoated Petri dish for 30 minutes. After the 30 minutes, the media was collected, and centrifuged at 190 g for 10 minutes to finally obtain the purified OPCs. 20.000 cells were incubated on a laminin-treated plate to perform uptake and differentiation experiments.

Up-take studies

Note: The fact that each vehicle has to be generated under different protocols made it impossible for us to administer them in the same concentration. To solve that, we added each vehicle in order to dispense similar particles counts, independently of the volume that was added to the culture. Red-Lp, Red-Np labelled with DiOC18 and Coumarin

respectively and Ne-Ex labelled with CM-DiL Celltracker were added to the OPCs and incubated for 24 hours in order to determinate the percentage of cells that were up-taking the particles. A total volume of 100 µl of Liposomes, 1 µl of Nanoparticles and 10 µl of Exosomes (100 µg of quantified protein) were administered to the culture. These volumes made a final number of 2.7 x $10^{5 \text{ or } 6}$ particles for all vehicles.

24 hours later, OPCs were fixed with 4% paraformaldehyde and labelled with Hoechst. Images were obtained with 20x magnification in a Nikon Eclipse80i (Nikon) and NIS elements AR 3.2 (Nikon) was used to capture the images. Fields were randomly selected. LSM 880 confocal microscopy (Zeiss) was used at a 60x magnification to obtain individual cell images. Positive and negative cells were counted and the average up-take levels quantified.

Differentiation studies

Similar to up-take studies, 219-Lp, E-Lp, 219-Np, E-Np, 219-Ex and Ne-Ex were administered to OPCs in the following concentrations: A total volume of 100 µl of liposomes, 1 µl of nanoparticles and 10 µl of exosomes (100 µg of quantified protein) were dispensed to the culture making a final concentration of 2.7 x $10^{5 \text{ or } 6}$ per well. Due to the low quantity of miR-219a-5p present in the cargo of exosomes, these vesicles were administered daily. A unique dose of liposomes and nanoparticles was administered at time zero. Three days after administration, RNA from OPC was isolated in order to perform gene expression studies and determine the degree of cell differentiation. RNA was isolated using Trizol LS protocol following the manufacturer´s instructions (#10296028 Thermo Fisher). Then, total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (#4368814, Thermo Fisher) following manufacturer's protocol in a Veriti Thermal Cycler (Applied Biosystems). Gene expression analysis of 2',3'-cyclic nucleotide 3' phosphodiesterase (*Cnpase*) myelin oligodendrocyte glycoprotein (*Mog*), myelin basic protein (*Mbp*) and proteolipid protein (*Plp1*) were performed in order to establish the differentiation degree of the cells, using KiCqStart SYBR Green qPCR ReadyMix and KiCqStart SYBR Green Primers (KCQS02 and KSPQ12012, Sigma). Ribosomal protein L13α (*Rpl13a*) and phosphoglycerate kinase 1 (*Pgk1*) were used as endogenous controls. CFX384 Thermal Cycler (Bio-Rad) was used to run the qPCR and raw data was processed in

Bio-Rad CFX Manager software. The calculation of relative expression was carried out with Excel software using the 2^{-DDCT} method [270].

Experimental Autoimmune Encephalomyelitis (EAE)

All animal procedures were approved by the Biodonostia Health and Research Ethics Committee (CEEA17_002).

Induction and clinical evaluation

10 weeks old C57BL/6 female mice were immunized with Hooke Kit™ MOG_{35-55}/CFA Emulsion PTX kit (#EK2110, Hooke Laboratories) as described in the manufacturer´s protocol. Briefly, animals were anesthetized with isoflurane 2% and 100 µl of MOG_{35-55} and Freund´s adjuvant emulsion were injected subcutaneously in two locations, administrating a total volume of 200 µl per animal. 2 and 24 hours after emulsion administration, 80 ng of Pertussis Toxin were administered to each animal. Animals were weighed and scored daily with the new clinical scale proposed by our group and defined in Chapter Two. The monitoring was performed in a blind manner in order to achieve objective scores.

Exosomes administration

100 µg of 219-Ex and Ne-Ex were administered 2 and 8 days after disease induction. To do this, mice were anesthetised with isoflurane 2% and 10 µl of exosomes (10 µg/µl) were administered to each animal in four doses, two per nostril, for 10 minutes.

Sample extraction

Animals were anesthetized with isoflurane 2% and cardiac puncture was carried out to obtain blood at final term. Additionally, spinal cords were extracted and fixed in PFA at 4% for 72 hours. Then spinal cords were stored at 4ºC in PBS with 0.01% of azide until Magnetic Resonance Imaging was carried out.

Plasma derived cytokine measurement

Blood samples obtained from cardiac puncture were centrifuged at 300 g for 15 minutes and plasmas were isolated. Plasma concentration of IL-10 IL-17A and TNFα were measured by a mouse high sensitivity T cell magnetic bead panel as previously described and following the manufacture´s protocol (MHSTMAG-70K Millipore) [289].

Then Bio-Plex MagPix (MerckMillipore) was used to run the samples and perform the analysis.

Magnetic Resonance Imaging

Magnetic Resonance Images (MRI) of mice spinal cord (animals with lowest clinical score per group; n=1) were acquired using a Bruker Biospec USR 117/16 MRI system interfaced to 4 transmit and 8 receive RF channels with a XYZ set of actively shielded gradients 750 mT/m and a slew rate of 6660 T/m/s. RF transmission and reception was achieved by using a transmit volumetric coil of 72 mm of i.d. (T1148V3), and receive surface coil (mouse brain surface coil, T11657V3) of approx. 2 cm diameter, both from Bruker Biospin GmbH (Ettlingen, Germany).

Tissue samples were immersed in PBS for imaging. After the acquisition of a series of scout images, Diffusion Weighted Images of the samples were acquired using a DtiStandard sequence for Buker software Paravision 6.0.1, with the following parameters: Spin-Echo DWI, 12 diffusion directions (d=4 ms, D=11 ms, b=1000 s mm^{-2}), 1 image with b=0 s mm^{-2}, echo time TE= 21 ms, repetition time TR=3000 ms, N Averages= 2, Image Matrix of (256x256) points with a Field-of view FOV= (25.6 x 25.6 mm), giving an in-plane resolution of (100 x 100 µm), and acquiring 60 consecutive slices of 0.2 mm thickness. Spectrometer bandwidth was set to BW = 66 kHz and the total scanning time resulted in 4h 9m 36s. The open source software DIPY (Diffusion Imaging in python), with local PCA denoising, was used for the processing of acquired DTI images, to obtain fractional anisotropy (FA) Further image analysis was performed with Image-J software, from NIH.

Statistical analysis

qPCR data relative expression was calculated by 2^{-DDCT} method. Mann Whitney test was used to analyse differences in the score of EAE animals and the significance of MRI fractional anisotropy of MRI images.

RESULTS AND DISCUSSION

Vehicles characterization

NTA was used to characterize the distribution in size and number of particles present in the samples. As shown in Figure 22 A, liposomes showed two main populations, the principal one at a size of 160 nm and a secondary one at 230 nm. Nanoparticles displayed a more homogeneous size distribution the main size being between 180 and 220 nm. In contrast, exosomes were the most heterogeneous sample the distribution being between 150 and 400nm. These results are in concordance with the genesis procedure of each vehicle. On the one hand liposomes and nanoparticles are synthetically generated in the laboratory with standard protocols to produce a homogeneous sample. On the other hand, exosomes are membrane-bound particles secreted by cells. They have been described to be different in size depending on their biogenesis [290]. Exosomes are described as the smallest vesicles; However, it must be noted that in many scenarios it can be difficult to separate those based exclusively on their size [152] indicating that our sample does not only contain exosomes, but also microparticles. These results were confirmed by electron microscopy (Figure 22, B) in which similar size vesicles can be found when compared to NTA.

When we analysed the cargo of microRNA in each vehicle, we determined that liposomes were able to encapsulate miR-219a-5p more efficiently than nanoparticles and these more than exosomes. With the particle counts data obtained from the NTA, we quantified the copies of microRNA per particle, determining that liposomes were the most enriched particles followed by nanoparticles and finally exosomes (Figure 22, C). In our hands, exosomes were the most time-comsuming vehicle to be generated. HEK293T cells needed to be grown for approximately 2 weeks before isolating exosomes, which took two extra days. On the other hand, liposomes and nanoparticles took less time and the synthesis protocol was more reproducible.

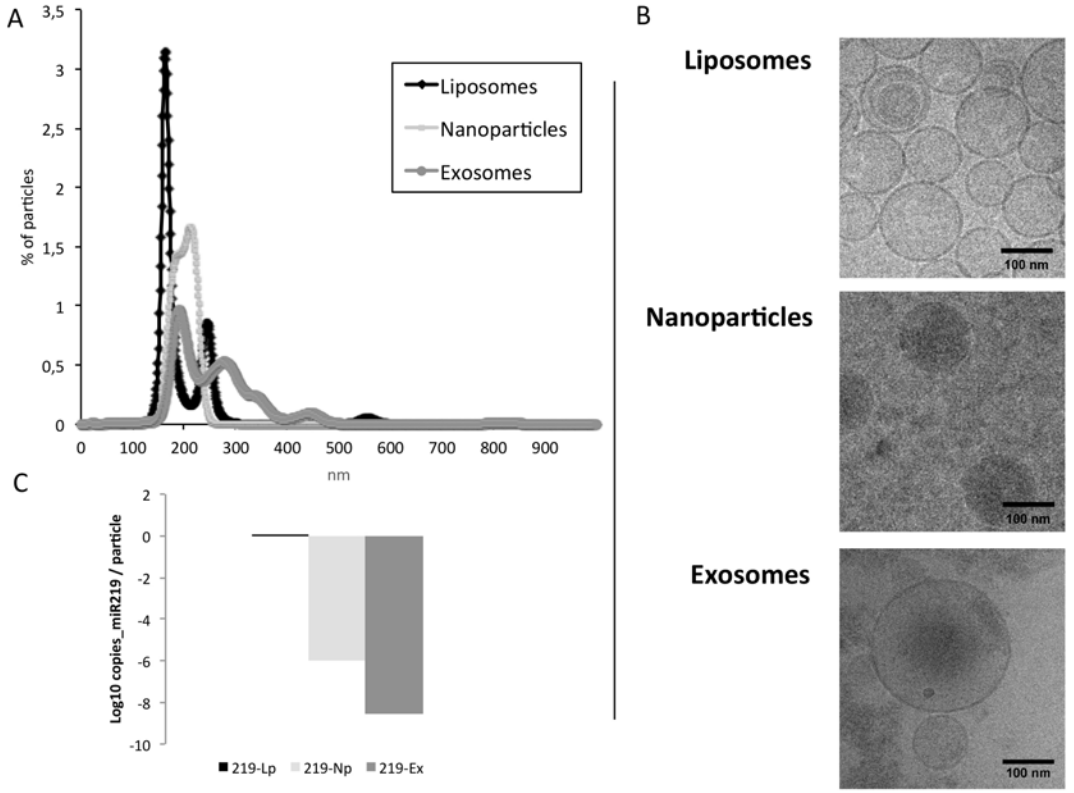


Figure 22: A) Nanoparticle Tracking Analysis (NTA) of liposomes, nanoparticles and exosomes. B) Cryo-TEM images of the three vehicles showing correlation in size with NTA. C) Levels of miR-219a-5p in liposomes, nanoparticles and exosomes. Liposomes are the most enriched vehicles followed by nanoparticles and finally exosomes.

Uptake and differentiation studies

Later, we wanted to test the ability of these vehicles to be taken up by OPCs. To do this, we labelled liposomes, nanoparticles and exosomes with DiOC18, Cumarin and CM-DiI respectively. 24 hours after administration, cells were fixed and microscopy images acquired determining that liposomes were taken up more efficiently by cells (95,83%, Stdv 5,9), followed by nanoparticles (83,98%, Stdv 5,1) and finally exosomes (61,48%, Stdv 2,8) (Figure 23, A and B). To study the potential of the three vehicles to induce OPC differentiation, a necessary step for remyelination, 219-Lp, 219-Np and 219-Ex were compared to their respective empty or non-enriched vehicles. Interestingly we saw that only exosomes were able to significantly increase the expression of myelin related genes (*Cnpase, Mbp, Mog* and *Plp1*) indicating a more differentiated state of the OPCs

(Figure 23C). However, our results indicate that exosomes, but neither liposomes nor nanoparticles, were able to induce OPC differentiation.

Interestingly, exosomes showed the lowest miR-219a-5p and up-take levels when compared to liposomes and nanoparticles but was the only vehicle able to induce OPC differentiation. Our initial idea was that liposomes and nanoparticles could be more appropriate microRNA delivery systems due to their homogeneity in composition and cargo and that their generation can be controlled. On the other hand, exosomes are biologically formed vesicles, which play an essential role in indirect intercellular communication and can transfer cytosolic proteins, lipids, metabolites and genetic material from one cell to another [252,253] indicating that other factors can be acting as helpers of the effect produced by miR-219a-5p. In fact, our results show that the biological complexity of exosomes, seems to be beneficial for OPC differentiation. Moreover, exosomes can be integrated in two different ways; by direct fusion with the plasma membrane or by endocytosis [291,292] demonstrating the higher ability of exosomes to integrate to the receptor cell. In addition, their cargo contain some machinery that favours microRNA processing such as Dicer, Ago2 or TRBP [293] . In relation to this, exosomes have shown to be efficient microRNA delivery systems for several diseases [294]. In order to understand further which microRNAs could be favouring the demonstrated effect, a microRNA profiling of exosome cargo was carried out using microarray technology. Surprisingly, miR-219a-5p was not detected by this array. This unexpected result questions the sensitivity of microarray technology, given that we had already confirmed the overexpression of miR-219a-5p in these exosomes. Nonetheless, to be sure that miR-219a-5p was present in enriched exosomes, we performed a qPCR experiment, which confirmed our previous ddPCR data (Ct value in qPCR = 27). Additionally, both ddPCR and qPCR demonstrated that 219-Ex contained between 30 and 100 times more miR-219a-5p when compared to Ne-Ex.

miR-219a-5p enriched exosomes improve EAE clinical evolution.

Once we had determined that exosomes were able to induce OPC differentiation, we wanted to go a step further and test their ability to improve EAE clinical score. Exosomes were intranasally administered as previously reported [177], to allow them to reach the CNS. Two doses were dispensed at days 2 and 8 after disease induction and the score was monitored daily. When analyzing the score, we could detect significant

differences at days 21, 22 and 25-29 when compared to the non-enriched Exosomes treated group. Interestingly, differences appeared after the disease peak, indicating that Exosomes could be promoting myelin regeneration (Figure 24, A). To confirm this, MRI images were obtained from spinal cords in mice (Figure 24, B). Fractional anisotropy is significantly increased in miR-219a-5p enriched exosomes treated animals when compared to non-enriched exosomes treated animals (p=0,001; Figure 24, C).

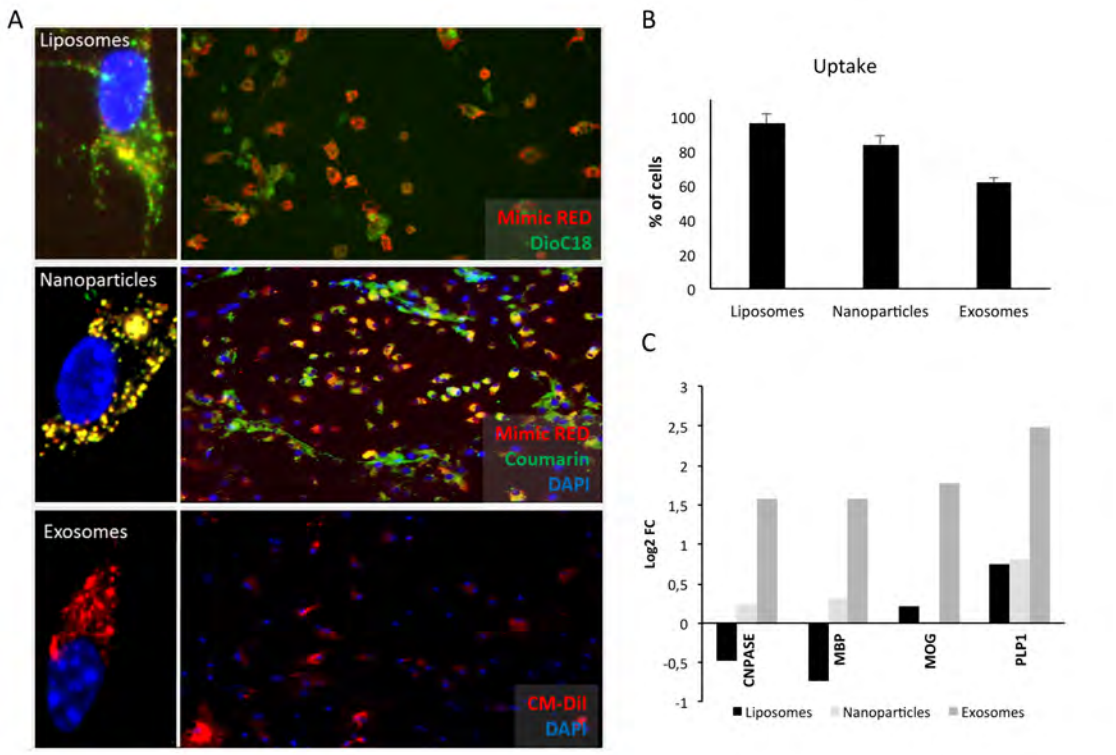


Figure 23: A) Uptake studies of liposomes, nanoparticles and exosomes. Liposomes containing DioC18 (green) and mimic-Red (red), nanoparticles containing Coumarin (green) and mimic-Red (red) and exosomes labelled with CM-DiL are shown. Individual cells are confocal microscopy images of representative samples. B) Percentage of OPCs that are able to take up each vehicle. Liposomes are the most efficient vehicle followed by nanoparticles and finally exosomes. C) Expression levels of myelin related genes in OPC cultures treated with each vehicle compared to the same empty vehicle. Exosomes are the only vehicle able to induce OPC differentiation.

Additionally, we analyzed the inflammatory patterns in plasma of EAE mice, but no significant differences were found in plasma derived cytokine levels between both groups (Figure 24, D). This result indicated that miR-219a-5p enriched exosomes were

not regulating the immune system of the animal supporting the idea that significant differences shown in the clinical score evolution could be related to a regeneration of the myelin.

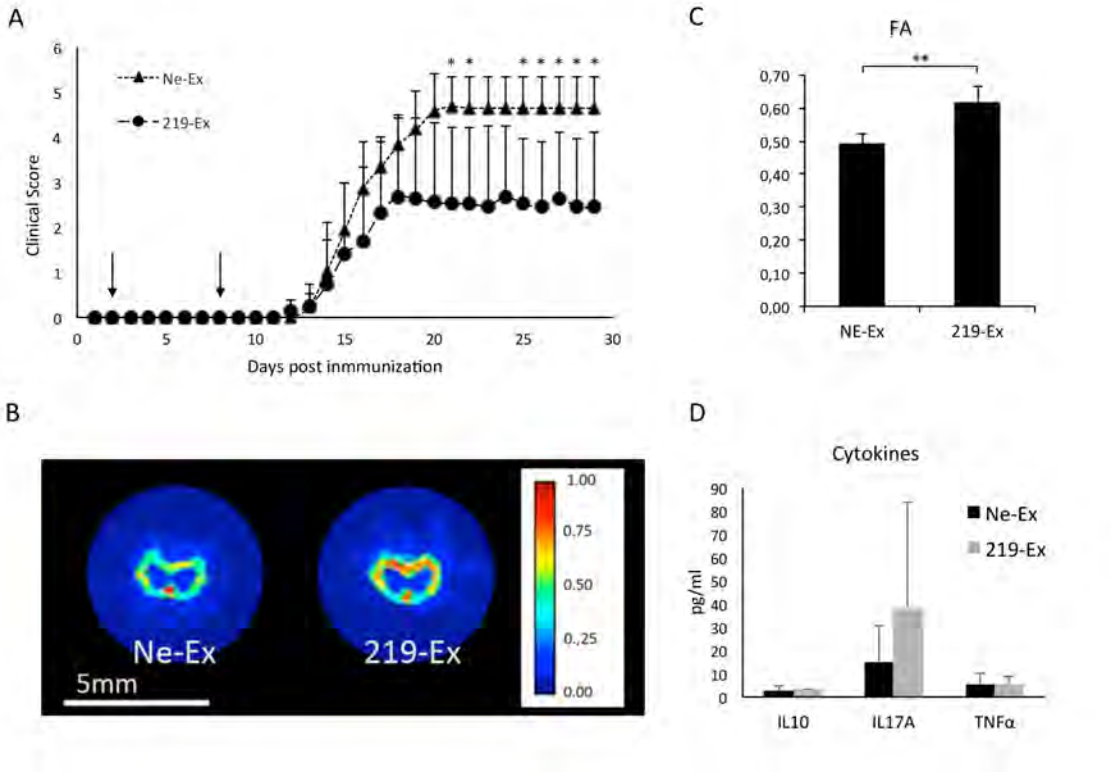


Figure 24: A) Clinical evaluation of animals treated with non-enriched exosomas (Ne-Ex) and with miR-219a-5p enriched exosomes (219-Ex). 219-Ex treated animals showed a significant decrease in the clinical evaluation after the disease peak (n=4). B) MRI of spinal cord of a Ne-Ex treated animal and a 219-Ex treated mouse showing the fractional anisotropy (FA). C) FA values of a section of the spinal cord of previous animals showing a decrease in FA values when treatment was Ne-Ex, indicating that remyelination is occurring. D) No significant differences between both groups in pro-inflammatory cytokines were found, indicating that the effect induced by exosomes was not related to an anti-inflammatory process.

CONCLUSION

Remyelination is a key aspect in MS pathology and a special effort is being made to promote this process. However, there is still no commercially available treatment to regenerate damaged myelin. Because of this, several strategies are being scrutinized. MicroRNAs have been postulated as a feasible and promising tool to induce OPC differentiation and therefore remyelination. However, microRNA delivery mechanisms to the CNS are still under study and the use of liposomes, nanoparticles and exosomes has been addressed in this work.

Although liposomes and nanoparticles were more enriched in miR-219a-5p and showed higher uptake levels than exosomes, those exosomes were surprisingly, the only delivery system able to induce OPC differentiation. However, as mentioned before, exosomes are biological delivery systems which contain other proteins, lipids and genetic material that can be integrated into the cell in several ways and that contain microRNA processing molecules, indicating that the efficiency of exosomes as microRNA delivery systems could be influenced by their biological context, making them more efficient delivery systems than liposomes or nanoparticles.

When exosomes were administered intranasally to the EAE models, they were able to decrease the clinical score, with no immuneregulation. In addition, significant differences were shown after the disease peak, indicating that they might be increasing the myelin production, which was also correlated with MRI results.

To conclude, miR-219a-5p has been identified as a necessary but not sufficient condition to induce remyelination. Additionally, miR-219a-5p enriched exosomes stimulate OPC differentiation and improve EAE clinical evolution opening a therapeutic approach for MS patients. In addition, this work shows that the use of exosomes as a microRNA delivery system for CNS diseases can be a promising and feasible tool.

GLOBAL DISCUSSION and

personal opinion

…with the idea of confirming if young rodent derived exosomes were able to reduce the clinical score of the EAE model".

Unfortunately we were not able to see an improvement in the clinical score of the animals after intranasal administration of young blood-derived exosomes. Moreover, we could not see a positive effect either in the cerebellar organotypic culture or in the OPC culture. When we tried to investigate the reason for the lack of effect of our young blood-derived exosomes, we discovered that they did not contain miR-219a-5p or other pro-remyelinating microRNAs.

At that point we decided to find out if miR-219a-5p was able, as previously described by several groups [228,231,233], to induce OPC differentiation. To do this, we chose a synthetic microRNA which was administered to the OPC and cerebellar organotypic cultures with lipofectamine. We determined that this synthetic microRNA was able to induce OPC differentiation in the OPC culture and to increase the generation of OPC cells in the cerebellar organotypic culture. These data made us think about the possibility of administering the microRNA to the EAE model.

However, and previous to continuing with this line of action, we wanted to understand the role that miR-219a-5p could be playing in patients' remyelination. For that reason, we analysed the level of miR-219a-5p in patients' blood. Although, we were not able to see significant differences between relapse and remission patients, we were able to detect a tendency in which miR-219a-5p is increased after a demyelinating insult. Further studies are needed to increase sample size, to study this process in more advanced stages of the disease and to isolate neuronal origin EVs by immunoprecipitation [295], but our results indicate that miR-219a-5p might be a key player in remyelination. Additionally, these preliminary results postulate miR-219 as a possible biomarker of the level of remyelination in MS patients.

These results reinforced our idea of administrating miR-219a-5p to the animal model in order to promote remyelination. Nevertheless, we had to decide which could be the best and most effective method of delivery. Direct administration was previously demonstrated to be an effective way to induce myelin regeneration [230,234]. However, we

considered that this was more a proof of concept rather than a real delivery system for MS patients. We thought that direct administration should be avoided in human therapy, especially if repetitive administrations have to be carried out (remembering that MS is distributed along space and time), therefore we selected a non-invasive delivery method. Several reports have been published indicating that intranasal administration of cells, molecules and exosomes could be an effective, non-invasive and secure method to reach the CNS [257,296–298] .

With this objective, miR-219a-5p should be encapsulated for protection it and to allow it to cross the BBB and reach the CNS to produce the expected effect. Viral vectors were discarded due to safety questions, such as a possible oncogenic transformation of the recipient cell. In addition they can stimulate the innate and adaptive immune responses reducing its effectivity [249]. Interestingly, liposomes and nanoparticles have been described as effective synthetic molecule delivery systems. In collaboration with CICbiomaGUNE and TECNALIA, liposomes and nanoparticles were respectively developed and loaded with the synthetic miR-219a-5p. In parallel, and thanks to collaboration with the University of Oxford, we generated exosomes enriched in miR-219a-5p.

Once the vehicles were characterized, we decided to test and compare the ability of liposomes, nanoparticles and exosomes to induce OPC differentiation. Interestingly, and although exosomes contained the lowest microRNA and uptake levels, it was the only vehicle able to induce OPC differentiation. In order to explain this, we should remember that exosomes are “biologically designed” to send information from one cell to another, making them, in our opinion, more efficient when compared to liposomes or nanoparticles. Additionally, they also contain other molecules such as proteins and genetic material that can interact with miR-219a-5p and increase its effect.

With these results, we decided to address if miR-219-a-5p enriched exosomes could be able to induce an improvement in the clinical evolution of the EAE animal after intranasal administration. Interestingly, we saw an improvement in the clinical score when compared to non-enriched exosomes indicating that the use of miR-219a-5p enriched exosomes could be a therapeutic strategy for MS patients.

Personal opinion

During the 5 years that I have worked in this project, several aspects have been discussed and studied. At this point I would like to give my personal opinion in relation to a) technical aspects and b) the use of microRNA delivery systems for demyelinating diseases and other neurodegenerative diseases.

Technical aspects - remyelination models

In a great number of works (including this one) the scientific community has shown it is able to promote myelin regeneration in several models. It is clear we are able to cure mice. However, nowadays there is still no remyelination strategy available for MS patients, indicating that the transfer of these results to the clinic is a difficult question. Animal models are just this, models that try to mimic the processes that take place in human pathologies. However, they have clear limitations regarding molecular differences or output deficiencies, among others.

Multiple sclerosis is a disease which mainly affects young adults and has a clear chronic component. Moreover, age has been associated with the disease evolution, as remyelination potential decreases with age. Therefore, I consider that one factor that should be taken into account when using remyelinating models is age. However, the models that have been used in this thesis and that are commonly used, are all derived from young animals. For example, OPCs and cerebellar slices are obtained from newborn mice, while the EAE model is performed in young mice. If we are trying to understand the remyelination process and the ability of some drugs to promote it, I believe that older models should be used. I recognize that obtaining OPCs from adult animals is a tricky question, but some efforts have been made in this direction [59]. Similar to this, cerebellar organotypic culture is more difficult to grow when derived from adult animals, but again, if we want to obtain more realistic data, this effort must be made. Finally, the EAE is a complex model, and the use of aged animals will help to mimick the process that takes place in MS patients, such as the decline of the remyelination ability.

As mentioned above, another key aspect in the use of remyelination models is their output. The better output, the more realistic results will be obtained. Regarding cerebellar organotypic culture, confocal microscopy analysis is the gold standard tool

for analysing the remyelination level in the model. Briefly, antibodies against myelin and axons are used and confocal images are obtained to quantify the colocalization percentage. However, image reproducibility must be high in order to compare one slice to another. We experienced difficulties performing reproducible immunofluorescence for myelin and axons due to the high variability of the culture so we decided to use other techniques. In a similar approach we used Western Blot to quantify total myelin and axon protein levels. Additionally, qPCR for myelin related genes were carried out. Although these techniques showed promising results, they do not show if the myelin is wrapping axons and if real remyelination is occurring. In order to solve that, in an ambitious approach in collaboration with CICbiomaGUNE, we decided to quantify the remyelination level by MRI. We obtained excellent images when slices were not cultured (Figure 25). However, after one week in culture, slices lost their thickness and it was extremely difficult to obtain proper images to quantify remyelination. This work led us to present two posters in international congresses. At this point, I would like to mention that, in our hands, the organotypic culture model is a really complex model and that obtaining reproducible data is difficult. Although it is a widely used model and many articles have been published including this model, after this work I contemplate that it is not an appropriate model for studying remyelination.

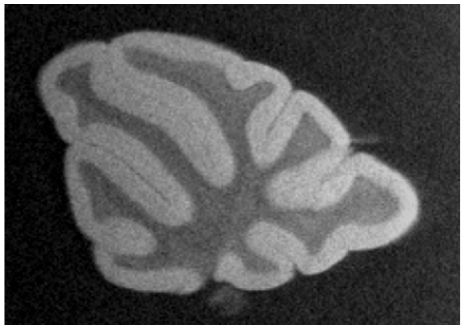


Figure 25: Cerebellar organotypic culture slice image obtained with MRI (Photo taken by Ander Egimendia)

To continue with the output of remyelination experimental models, as widely explained in Chapter Two, we noticed that the EAE animal model clinical score could be improved. I wanted to highlight that the way data are obtained from this model is crucial to obtain objective, representative and realistic results of the experiments. To facilitate the data acquisition and analysis process, we tried to improve the clinical evaluation system and developed a mobile application (that is at the moment being

evaluated by a software company). Nevertheless, this is a secondary measurement of the degree of pathophysiological damage to the animals and therefore, histological analyses are commonly performed. In this case we decided to use MRI to characterize the remyelination degree at the end point. But I consider that producing and analysing MRI images throughout the evolution of the disease is the best way of characterizing the remyelination level of an animal, allowing the evaluation of this animal during the experiment and, in this way, reducing the number of mice that are needed per group.

To conclude this section, I would like to mention that during this work, I have changed my opinion regarding the models and their output methodologies. This evolution is reflected during this work, especially in Chapters One and Four. For example, organotypic cultures have not been used in Chapter Four, and the EAE induction methodology has been modified to obtain a more homogenous model.

Therapeutic use of microRNA delivery systems

It has been proposed that the use of an immunomodulatory or immunoregulatory drug in combination with a remyelination promoter could be an effective way of treating MS patients. In this work, we have proposed that miR-219a-5p is an efficient remyelination inductor and that the encapsulation of this microRNA in exosomes could be a feasible strategy to promote remyelination.

On the other hand, liposomes and nanoparticles did not prove to be effective delivery systems in the OPC culture. However, this does not mean that they could not be effective in this process. Liposomes and nanoparticles could be synthetized in several ways and their components and their proportions can be modified in order to obtain more efficient vehicles. Additionally, the location of the microRNA were it is included in the vehicle could influence their effectiveness, as they have been shown to be around and inside liposomes and nanoparticles. In order to select the most efficient formulation of vesicles as microRNA delivery systems, we based on the expertise of our collaborators, their preliminary results and also on previous literature. However, and although these vehicles were not effective in our task, further studies should be made as they have clear advantages when compared to exosomes. For instance, their synthesis is well characterized and very reproducible and the components are perfectly known facilitating the approval of their use as therapeutic agents or mediators.

Regarding the use of microRNAs as remyelinating promoters, some authors have postulated that the combination of more than one microRNA could be a more effective way of inducing OPC differentiation and therefore remyelination. More concretely, miR-219a-5p in combination with miR-338 and miR-138 demonstrated they induce remyelination more efficiently than miR-219a-5p alone [230,231]. However, in our experience miR-219a-5p on its one was more effective than the combination of these microRNAs (data not shown) and this is why we used only miR-219a-5p. But further studies could be made combining other microRNAs or other molecules with miR-219a-5p in order to obtain more powerful results.

As previously mentioned, we demonstrated that miR-219a-5p enriched exosomes were able to induce remyelination in the EAE animal model after intranasal administration. However, we did not prove if exosomes reached the CNS. Although some papers have been published indicating that intranasally administrated exosomes were able to reach the CNS [299] we would have liked to confirm it in our laboratory. In collaboration with CICbiomaGUNE, we are trying to label exosomes with a contrast agent that can be detected using MRI. This technique will aid the tracking exosomes in time to confirm if they are reaching the CNS and in what proportion. Another aspect that should also be kept in mind is if, once the vehicles reach the CNS, they have tropism to OPCs (which are the target cells of this therapy) or on the contrary, they become fused with other cell types. The exosomes that have been used in this work did not contain any factor that could aid them to target OPCs and therefore I consider that exosomes are targeting all cell types, This might produce side effects which should be addressed in further studies. Although we obtained positive results, I think that exosomes could be directed to OPCs by using antibodies, in this way improving the efficacy and reducing side effects, making this therapeutic strategy more effective and less risky.

The EVs isolation method is also a relevant aspect to be mentioned, as several methods can lead to different EVs types. Although great effort has been made in order to standardize isolation techniques [300], there is still controversy in which is the most efficient method. For example, conditioned media has demonstrated to induce modification in cells after its administration *in vitro* [301], but the isolation of EVs by ultracentrifugation of the conditioned media was also effective [184], highlighting that very different techniques can lead to positive results. In Chapter One, we decided to

replicate some aspects of a previous work and therefore we used Exoquick TC. However, in Chapters Three and Four we decided to use the most efficient method to our hand [151], which consists in differential centrifugation steps.

Another thing to consider when speaking about exosomes as remyelination mediators is the source of the therapeutic exosomes. EVs are, from a biological point of view, complex vehicles that contain a large number of components. In this work we have focused exclusively on microRNAs, but it has been suggested that they are only 1% of the cargo [302]. Additionally, as we have shown in Chapter One, the environment can modify the cargo of vesicles, complicating the isolation of reproducible EVs batches. Therefore, the source of the EVs and the conditions under which they are obtained could influence their cargo and their ability to modify target cells. In this sense, established cell lines or cells isolated from the patient or a donor can be used to isolate EVs. Biofluids such as plasma or urine are also an alternative. It is not clear which source is the most efficient and each one has its detractors. I consider that the use of established cell lines facilitates the isolation of EVs under reproducible conditions. In Chapter Four we decided to use HEK cells as an easy to grow and manage cell line. However, I think that using neural derived cell types could be more effective as they may have tropism to the CNS. At the moment we are obtaining miR-219a-5p enriched exosomes from neural derived cell lines.

Additionally, in our approximation we decided to modify HEK cells by bioengineering techniques to obtain exosomes enriched in miR-219a-5p. However, non-modified EVs have shown promising results [182,257]. In fact, there are several clinical trials recruiting patients in which the ability of allogeneic mesenchymal stem cell derived exosomes in acute ischemic stroke or the effect of plasma derived exosomes on cutaneous wound healing will be addressed [303,304]. Nevertheless, the modification of the cargo of EVs by bioengineering techniques is an interesting and promising field in EV-mediated therapies and I consider that it might be a more effective treatment method as our data demonstrate. In addition to microRNAs, vesicles can also be loaded with small compounds and drugs with anti-inflammatory effects. As an example, curcumin loaded exosomes demonstrated they induce neuroprotection [187]. Finally, EVs can be modified to express membrane receptors of the target cell, in this way increasing the uptake by the target cell and decreasing off-target bindings [197], as previously mentioned.

To conclude, EVs have demonstrated they are key players in myelin regeneration and the applications that EVs could have in the stimulation of remyelination in pathological states are many [139]. As we have mentioned, remyelination therapies are still not available and the use of EVs is becoming a promising and feasible method to induce myelin restoration, in this way decreasing neurodegeneration and therefore, increasing patients' outcome. In *Figure 26* we summarize some approaches that can be performed in order to obtain therapeutic EVs. Our knowledge about the therapeutic potential of EVs is just beginning and an exciting future awaits us…

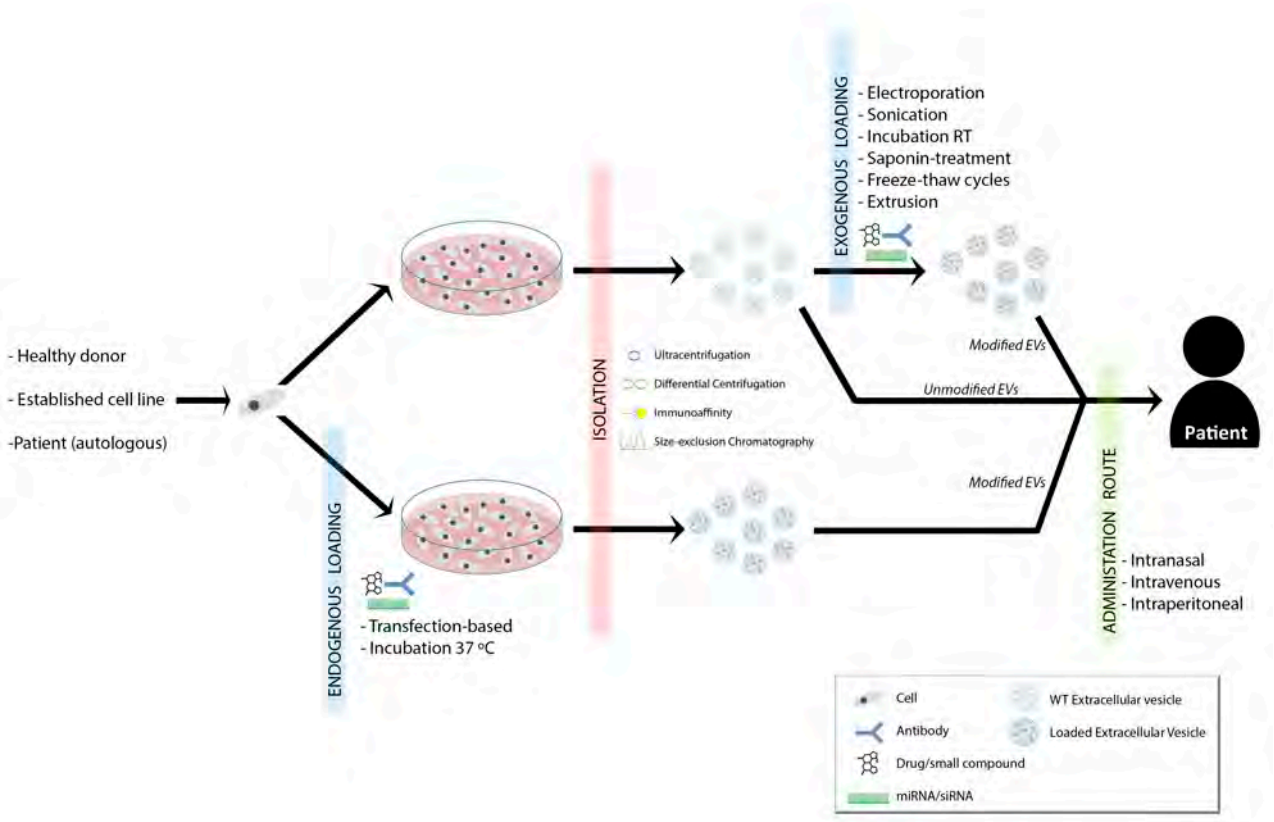


Figure 26: Summary of proposed therapeutic approaches for demyelinating diseases. Established cell lines, donor- or patient-derived cells are isolated and grown. EVs can be loaded with drugs/small compounds, miRNAs/siRNAs and/or surface antibodies, which provide new options in remyelination therapy. The loading can be performed during the cell culture (endogenous loading) or once EVs are isolated (exogenous loading)(reviewed in [305]). This might depend on the strategy and purpose of the therapy [306]. Therapeutic EVs can be isolated by ultracentrifugation, differential centrifugation,

immunoaffinity or size-exclusion chromatography [307]*. Finally, EVs could be administered to the patient intranasally, intraperitoneally or intravenously and again, this is something that will vary according to the therapeutic strategy.*

CONCLUSIONS

1. Environmental conditions affect the cargo of blood-derived exosomes, modifying therefore their functions.
2. MiR-219a-5p induces OPC differentiation in primary cells and activates remyelination in the cerebellar organotypic culture.
3. MiR-219a-5p is a key factor in the cargo of young blood rodent derived pro-remyelinating exosomes to induce OPC differentiation and remyelination in the EAE animal model.
4. The new EAE scale and the mobile application facilitate the acquisition of robust, reproducible and more objective data when monitoring EAE clinical evaluation.
5. MiR-219a-5p is enriched in the EV fraction derived from plasma when compared to total plasma.
6. MiR-219a-5p could play a role in the remyelination process after relapse in MS patients, being a possible biomarker of the remyelination stage.
7. Liposomes and Nanoparticles are more efficient encapsulating miR-219a-5p and being up-taken by OPCs when compared to exosomes.
8. MiR-219a-5p enriched exosomes is the only tested vehicle able to induce OPC differentiation.
9. MiR-219a-5p enriched exosomes are able to induce EAE clinical improvement when intranasally administered to the mice.
10. MiR-219a-5p enriched exosomes could be a promising and feasible therapeutic strategy to promote remyelination in MS patients.
11. Exosomes could be a possible microRNA delivery system for other neurodegenerative diseases.

SUMMARY OF PUBLICATIONS

Articles related to this work (PDFs in appendix section)

- **Osorio-Querejeta I,** Alberro A, Muñoz-Culla M, Mäger I, Otaegui D. 2018 Therapeutic potential of Extracellular Vesicles for demyelinating diseases; Challenges and opportunities. Front. Mol. Neurosci. 11, 434.
- **Osorio-Querejeta I**, Sáenz-Cuesta M, Muñoz-Culla M, Otaegui D. 2017. Models for Studying Myelination, Demyelination and Remyelination. NeuroMolecular Med.
- Sáenz-Cuesta M, Arbelaiz A, Oregi A, Irizar H, **Osorio-Querejeta I**, Muñoz-Culla M, Banales JM, Falcón-Pérez JM, Olascoaga J, Otaegui D. 2015. Methods for extracellular vesicles isolation in a hospital setting. Front Immunol 6.
- Sáenz-Cuesta M, **Osorio-Querejeta I**, Otaegui D. 2014b. Extracellular vesicles in multiple sclerosis : what are they telling us ? Front Cell Neurosci 8:1–9.

Articles related to this work (Submitted or in preparation):

- **Osorio-Querejeta I,** Sáenz-Cuesta M, Eguimendia E, Oregi A, Sepúlveda L, Irizar H, Alberro A, Llarena I, Aiastui A, Muñoz-Culla M, Otaegui D. MiR-219a-5p is a key factor in the cargo of pro-remyelinating exosomes. **UNDER REVIEW** in Molecular Therapy-Nucleic Acids
- **Osorio-Querejeta I,** Carregal S, Aierdi A, Mäger I, Nash L, Wood M, Eguimendia A, Alberro A, Iparraguirre I Llarena I Muñoz-Culla M, Ramos P, Otaegui D. Liposomes, Nanoparticles and Exosomes as pro-remyelinating microRNAs delivery systems in primery oligodendrocyte precursor cells and EAE mice. **IN PREPARATION**
- **Osorio-Querejeta I,** Sáenz-Cuesta M, Oregi A, Alberro A, Iparraguirre I, Moles L, Muñoz-Culla M, Otaegui D. Evaluating the Experimental Autoimmune Encephalomyelitis mice model. New insights into an old story. **IN PREPARATION**

Articles published during this work but not directly related to it:

- Sáenz-Cuesta M, Alberro A, Muñoz-Culla M, **Osorio-Querejeta I,** Fernandez-Mercado M, Lopetegui I, Tainta M, Prada A, Castillo-Triviño T, Falcon-Perez JM, Olascoaga J, Otaegui D. 2018. The first dose of Fingolimond affects Circulating Extracellular Vesicles in Multiple Sclerosis Patients. Int J Mol Sci.

- Muñoz-Culla, M., Irizar, H., Gorostidi, A., Alberro, A., **Osorio-Querejeta, I**., Ruiz-Martínez, J., … Otaegui, D. (2017). Progressive changes in non-coding RNA profile in leucocytes with age. *Aging*.
- Irizar H, Muñoz-Culla M, Sáenz-Cuesta M, **Osorio-Querejeta I**, Sepúlveda L, Castillo-Triviño T, Prada A, Lopez de Munain A, Olascoaga J, Otaegui D. 2015. Identification of ncRNAs as potential therapeutic targets in multiple sclerosis through differential ncRNA – mRNA network analysis. BMC Genomics 16:1–15.
- Muñoz-Culla M, Irizar H, Sáenz-Cuesta M, Castillo-Triviño T, **Osorio-Querejeta I**, Sepúlveda L, López de Munain A, Olascoaga J, Otaegui D. 2016. SncRNA (microRNA & snoRNA) opposite expression pattern found in multiple sclerosis relapse and remission is sex dependent. Sci Rep 6:20126:20126.
- Sáenz-Cuesta M, Irizar H, Castillo-Triviño T, Muñoz-Culla M, **Osorio-Querejeta I**, Prada A, Sepúlveda L, López-Mato MP, de Munain AL, Comabella M, Villar LM, Olascoaga J, Otaegui D. 2014a. Circulating microparticles reflect treatment effects and clinical status in multiple sclerosis. Biomark Med 8:653–61.

Poste presentation related to this work:

- **Iñaki Osorio-Querejeta**, Ander Eguimendia, Ainhoa Alberro, Laura Moles, Leire Iparraguirre, Susana Carregal-Romero, Irantzu Llarena, Leslie Nash, Lucia Sepúlveda, Carlos San José, Matthew JA Wood, Imre Mäger, Pedro Ramos-Cabrer, Maider Muñoz-Culla, David Otaegui. MiR-219 enriched exosomes decrease experimental autoimmune encephalomyelitis symptoms after intranasal administration. ECTRIMS 2018, Berñin, Germany. 10-12 October 2018.

- **Iñaki Osorio-Querejeta**, Ana Ayerdi , Susana Carregal-Romero, Leslie Nash, Ainhoa Alberro, Leire Iparraguirre, Irantzu Llarena, Imre Mäger, Matthew JA Wood, Pedro Ramos-Cabrer, Maider Muñoz-Culla, David Otaegui. Nanoparticles, Liposomes and Exosomes as microRNA delivery systems for Neurodegenerative disease; Remyelination inductors in Multiple Sclerosis. ISEV 2018. Barcelona Spain. 2-6 May 2018.

- **Iñaki Osorio-Querejeta**, Irantzu Llanera, Maider Muñoz-Culla, Ander Egimendia, Pedro Ramos-Cabrer, David Otaegui. Ultra-High resolution MRI and confocal fluorescence microscopy for the in vitro study of remyelination processes; EMIM 2017; Collogne, Germany, 5-7 April 2017

- **Osorio-Querejeta I**., Llarena I., Muñoz-Culla M., Ramos-Cabrer P., Otaegui D. Remyelination studies with confocal fluorescence microscopy; SPAOM2016; Bilbao, Spain, 5-7 October 2016

- **Osorio-Querejeta I**.ǂ, Muñoz-Culla M. ǂ, Alberich M., de la Cuesta M., Gorostidi A., Castillo-Triviño T., Olascoaga J., Otaegui D. Sequencing of potential causative genes from a GWAS study in multiple sclerosis familial cases. Negative results. ECTRIMS 2015; Barcelona, Spain, 7-10 October 2015

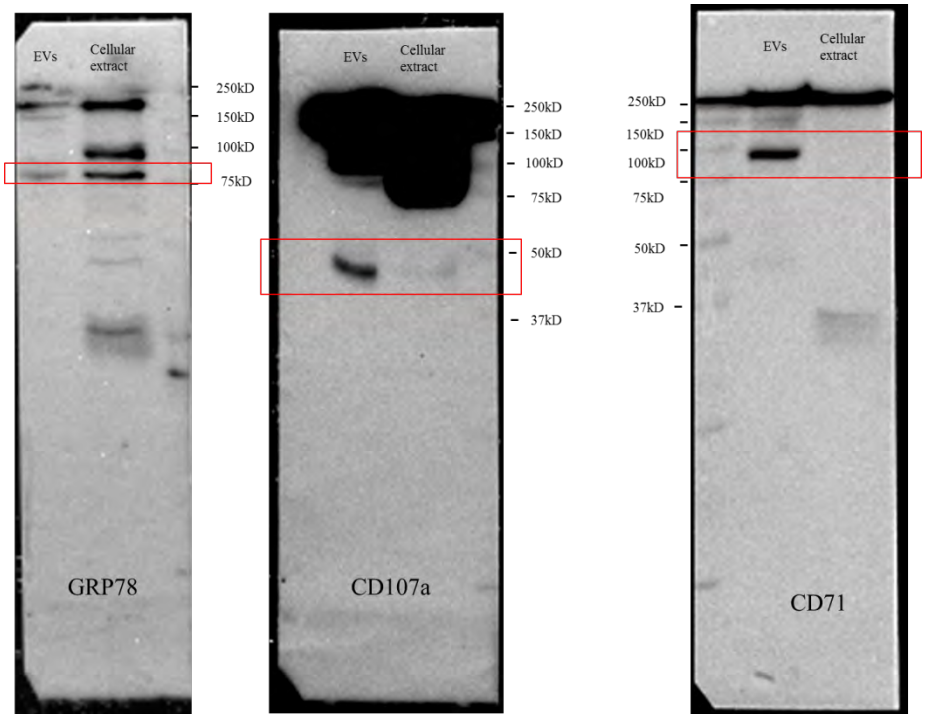
- **Osorio-Querejeta Iñaki**, Saenz-Cuesta Matías, Oregi Amaia, Muñoz-Culla Maider, Olascoaga Javier, Otaegui-Bichot David. Can Exosomes form Young blood promote remyelination? First Iberian Meeting of Extracellular Vesicles. Porto, Portugal, 30 September 2015

- **Osorio-Querejeta I**, Sáenz-Cuesta M, Gago B, Irizar H, Muñoz-Culla M, Castillo-Triviño T, Prada A, San José C, López de Munain A, Olascoaga J, Otaegui D. Characterization of blood Microparticles in an Experimental Autoimmune Encephalitis model. Neurogune 2013. Bilbao (Spain). 8 October 2013

SUPPLEMENTARY MATERIAL

CHAPTER ONE: MiR-219a-5p is a key factor in the cargo of exosomes to induce remyelination.

Supplementary Figure 1: Full-length gels of the Western Blot represented in Figure 13. Red squares represent the cropped areas shown in the Figure 13 for GRP78, CD107a and CD71.



Supplementary Table 1: Experimental autoimmune encephalomyelitis clinical score. Modified from Hooke Laboratories.

EAE scoring (modified from Hooke´s laboratories)	
Score	**Clinical observations**
0	No obvious changes in motor function compared to non-immunized mice. When picked up by base of tail, the tail has tension and is erect. Hind legs are usually spread apart. When the mouse is walking, there is no gait or head tilting.
0,5	Tip of tail is limp. When picked up by base of tail, the tail has tension except for the tip. Muscle straining is felt in the tail, while the tail continues to move.
1	Limp tail.

	When picked up by base of tail, instead of being erect, the whole tail drapes over finger. Hind legs are usually spread apart. No signs of tail movement are observed.
1,5	Limp tail and hind leg inhibition. When picked up by base of tail, the whole tail drapes over finger. When the mouse is dropped on a wire rack, at least one hind leg falls through consistently. Walking is very slightly wobbly.
2	Limp tail and weakness of hind legs. When picked up by base of tail, the legs are not spread apart, but held closer together. When the mouse is observed walking, it has a clearly apparent wobbly walk. One foot may have toes dragging, but the other leg has no apparent inhibitions of movement. - OR - Mouse appears to be at score 0.0, but there are obvious signs of head tilting when the walk is observed. The balance is poor.
2,5	Limp tail and dragging of hind legs. Both hind legs have some movement, but both are dragging at the feet (mouse trips on hind feet). - OR - No movement in one leg/completely dragging one leg, but movement in the other leg. - OR - EAE severity appears mild when picked up (as score 0.0-1.5), but there is a strong head tilt that causes the mouse to occasionally fall over.
3	Limp tail and complete paralysis of hind legs (most common). - OR - Limp tail and almost complete paralysis of hind legs. One or both hind legs are able to paddle, but neither hind leg is able to move forward of the hind hip. - OR - Limp tail with paralysis of one front and one hind leg. - OR - ALL of: Severe head tilting, Walking only along the edges of the cage, Pushing against the cage wall,

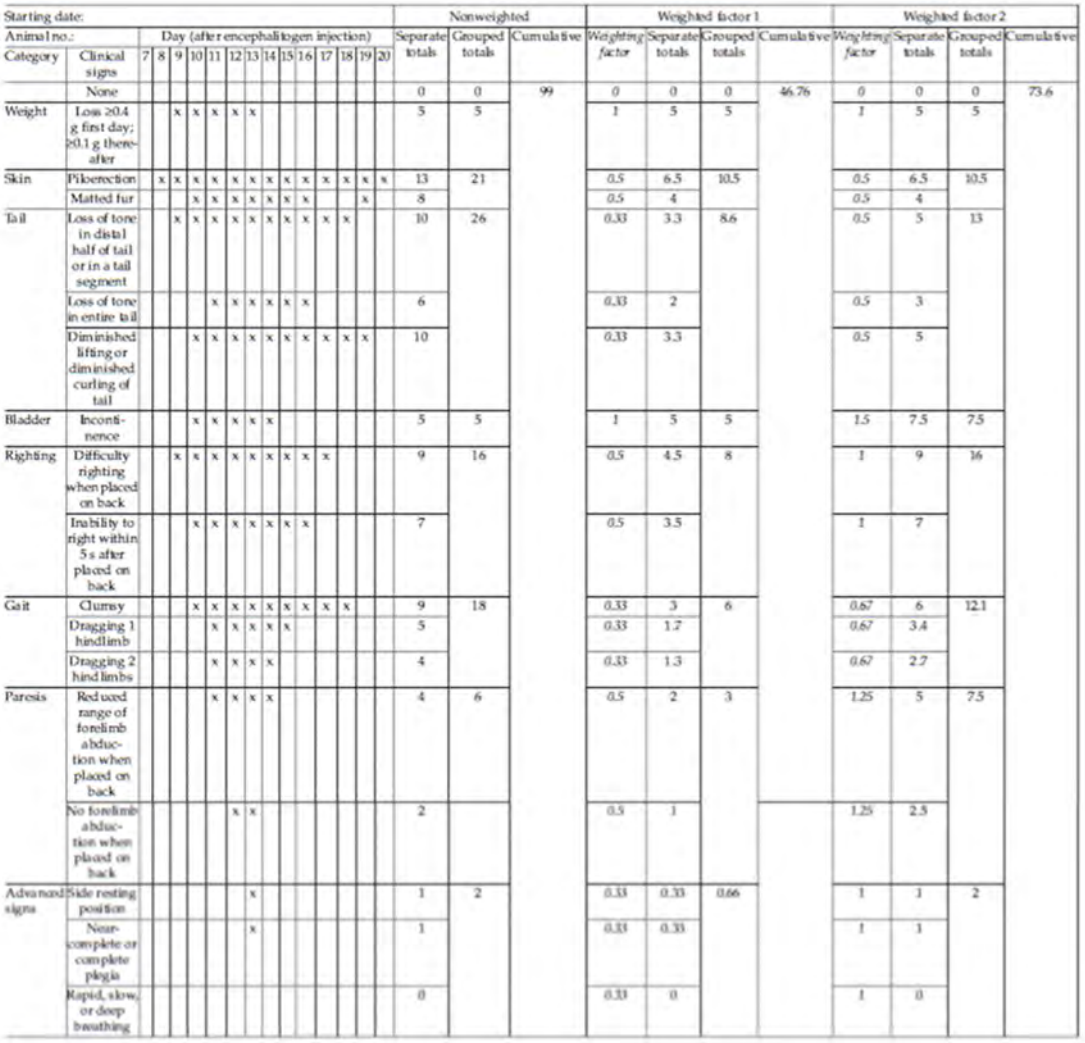
	Spinning when picked up by base of tail.
3,5	Limp tail and complete paralysis of hind legs. In addition to: Mouse is moving around the cage, but when placed on its side, is unable to right itself. Hind legs are together on one side of body. - OR - Mouse is moving around the cage, but the hind quarters are flat like a pancake, giving the appearance of a hump in the front quarters of the mouse.
4	Limp tail, complete hind leg and partial front leg paralysis. Mouse is minimally moving around the cage but appears alert and feeding. Often euthanasia is recommended after the mouse scores 4.0 for 2 days. However, with daily s.c. fluids some mice can recover to 3.5 or 3.0. When the mouse is euthanized because of severe paralysis, a score of 5.0 is entered for that mouse for the rest of the experiment.
4,5	Complete hind and partial front leg paralysis, no movement around the cage. Mouse is not alert. Mouse has minimal movement in the front legs. The mouse barely responds to contact. Euthanasia is recommended. When the mouse is euthanized because of severe paralysis, a score of 5.0 is entered for that mouse for the rest of the experiment.
5	Mouse is spontaneously rolling in the cage (euthanasia is recommended). -OR- Mouse is found dead due to paralysis -OR- Mouse is euthanized due to severe paralysis

CHAPTER TWO: Development of a new EAE clinical score and a mobile application to monitor the model.

Previous clinical scores used to evaluate EAE animals are shown. In Figure 27, the most basic scale to monitor EAE animals is shown. In supplementary material of Chapter One, Hookes laboratory clinical evolution can be found. This is an improvement of the score shown in Figure 27. In Figure 28 the scale proposed by Emerson et al can be found [278].

Score	Signs	Description
0	Normal behavior	No neurological signs.
1	Distal limp tail	The distal part of the tail is limp and droopy.
1.5	Complete limp tail	The whole tail is loose and droopy.
2	Righting reflex	The mouse can hang on to cage with its hind legs but it don't show fluently movements and it begins to show weakness signs.
2.5	No righting reflex	It's no longer able to hang on to cage with its hind legs. When riding it shows lopsided and with no fluently movements.
3	Beginning of Ataxia	Start dragging the hind legs retaining the ability to move.
3.5	Severe Ataxia	Drag the 2 legs and has difficulty moving its hind legs but show remnant movement.
4	Early Paralysis	The mouse has paralyzed the entire posterior region. Dragging the hind legs is unable to propel.
5	Full Paralysis	The mouse can't move its legs at all, it looks thinner and emaciated.
6	Moribund/ Death	

Figure 27: The most basic scale used to evaluate clinical evolution of EAE animals.



Starting date:																Nonweighted			Weighted factor 1				Weighted factor 2			
Animal no.:		Day (after encephalitogen injection)														Separate totals	Grouped totals	Cumulative	*Weighting factor*	Separate totals	Grouped totals	Cumulative	*Weighting factor*	Separate totals	Grouped totals	Cumulative
Category	Clinical signs	7	8	9	10	11	12	13	14	15	16	17	18	19	20											
	None															0	0	99	*0*	0	0	46.76	*0*	0	0	73.6
Weight	Loss ≥0.4 g first day; ≥0.1 g thereafter			x	x	x	x	x								5	5		*1*	5	5		*1*	5	5	
Skin	Piloerection	x	x	x	x	x	x	x	x	x	x	x	x	x	x	13	21		*0.5*	6.5	10.5		*0.5*	6.5	10.5	
	Matted fur				x	x	x	x	x	x	x			x		8			*0.5*	4			*0.5*	4		
Tail	Loss of tone in distal half of tail or in a tail segment			x	x	x	x	x	x	x	x	x	x			10	26		0.33	3.3	8.6		*0.5*	5	13	
	Loss of tone in entire tail					x	x	x	x	x	x					6			0.33	2			*0.5*	3		
	Diminished lifting or diminished curling of tail				x	x	x	x	x	x	x	x	x	x		10			0.33	3.3			*0.5*	5		
Bladder	Incontinence				x	x	x	x	x							5	5		*1*	5	5		*1.5*	7.5	7.5	
Righting	Difficulty righting when placed on back			x	x	x	x	x	x	x	x	x				9	16		*0.5*	4.5	8		*1*	9	16	
	Inability to right within 5 s after placed on back				x	x	x	x	x	x	x					7			*0.5*	3.5			*1*	7		
Gait	Clumsy				x	x	x	x	x	x	x	x	x			9	18		0.33	3	6		*0.67*	6	12.1	
	Dragging 1 hindlimb					x	x	x	x	x						5			0.33	1.7			*0.67*	3.4		
	Dragging 2 hind limbs					x	x	x	x							4			0.33	1.3			*0.67*	2.7		
Paresis	Reduced range of forelimb abduction when placed on back					x	x	x	x							4	6		*0.5*	2	3		1.25	5	7.5	
	No forelimb abduction when placed on back						x	x								2			*0.5*	1			1.25	2.5		
Advanced signs	Side resting position							x								1	2		0.33	0.33	0.66		*1*	1	2	
	Near-complete or complete plegia							x								1			0.33	0.33			*1*	1		
	Rapid, slow, or deep breathing															0			0.33	0			*1*	0		

Figure 28: Emerson et al proposed this clinical evaluation system in 2009. Modified from [278]

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APPENDIX: Publications

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
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Extracellular vesicles in multiple sclerosis: what are they telling us?

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Extracellular vesicles (EVs) are membrane-bound particles secreted by almost all cell types. They are classified depending on their biogenesis and size into exosomes and microvesicles or according to their cell origin. EVs play a role in cell-to-cell communication, including contact-free cell synapsis, carrying active membrane proteins, lipids, and genetic material both inside the particle and on their surface. They have been related to several physiological and pathological conditions. In particular, increasing concentrations of EVs have been found in many autoimmune diseases including multiple sclerosis (MS). MS is a central nervous system (CNS) demyelinating disease characterized by relapsing of symptoms followed by periods of remission. Close interaction between endothelial cells, leukocytes, monocytes, and cells from CNS is crucial for the development of MS. This review summarizes the pathological role of EVs in MS and the relationship of EVs with clinical characteristics, therapy, and biomarkers of the disease.

Keywords: extracellular vesicle, exosomes, microvesicle, multiple sclerosis, biomarker, therapy

WHAT ARE EXTRACELLULAR VESICLES?

Extracellular vesicles (EVs) are membrane-bound particles coming from inside a cell or formed directly from its membrane, and excreted to the extracellular medium, that carry information whose function is cell-to-cell communication without direct contact. They play a role in physiological and pathological conditions, being released during cell activation, stress, and apoptosis. Specifically, these vesicles carry proteins, lipids, and genetic materials such as DNA, RNA, and miRNA, producing genotypic (Waldenström et al., 2012) and phenotypic (van der Vos et al., 2011) modifications in the recipient cell. This is facilitated by the receptors on the surface of the EV membrane that allow the target cell to identify the vesicles and interact with them (Choudhuri et al., 2014).

EVs CLASSIFICATION

BIOGENESIS

Though there are several ways of classifying EVs, the main division in nomenclature is based on biogenesis. Those formed inside multivesicular bodies and released extracellularly upon fusion of these bodies with the plasma membrane are called exosomes (Théry et al., 2009). Their main characteristic is to have a uniform size of between 30 and 150 nm, making them the smallest EVs. On the other hand, those known as microparticles (MP), microvesicles (MV), or ectosomes come from the modification of the cell membrane after external or internal stimuli, leading to a softening of the membrane-adjacent structure and allowing evagination and vesicle formation followed by fission on the connecting membrane stalks until their full detachment. These MV/MPs vary greatly in size, ranging from 0.3 to 1 μm in diameter (Mause and Weber, 2010; Frey and Gaipl, 2011; Lai and Breakefield, 2012). However, the current trend is to call the entire set EVs, the term used by the newly formed International Society of Extracellular Vesicles (Witwer et al., 2013).

In this review, we follow this trend, using the term EVs to refer to all vesicles; we note, however, that specifically in multiple sclerosis (MS) related-research most studies refer to them as MPs or MVs.

CELL ORIGIN

Extracellular vesicles have been also classified as a function of their cell origin depending on the parental cell from which they arose, so far the most studied being those obtained from circulating cells in peripheral blood. Each cell has characteristic markers on its membrane enabling subsequent identification of the EV, e.g., as erythrocyte-, leukocyte-, platelet-, endothelial-, or monocyte-derived. Further, studies focusing on central nervous system (CNS)-derived EVs have described neural stem cell-, neuron-, astrocyte-, microglia-, and oligodendrocyte-derived vesicles (Lai and Breakefield, 2012) with the goal of finding markers that may reflect CNS status, since they can be detected remote from the site of release after cell activation.

TECHNIQUES FOR STUDYING EVs

The study of EVs is not straightforward, particularly with respect to isolation and characterization due to their small size and the low concentrations found in human fluids. Further, although efforts have been made to unify criteria in EV research (Robert et al., 2008; Dey-Hazra et al., 2010; Lacroix et al., 2012a; Witwer et al., 2013), they are not yet clearly established, making it difficult to compare

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalitis; EEV, endothelial-derived EV; EV, extracellular vesicle; LEV, leukocyte-derived EV; MEV, monocyte/microglia-derived EV; MP, microparticle; MS, multiple sclerosis; MV, microvesicle; PEV, platelet-derived EV.

studies. Differences derived from centrifugation protocols, fluorochrome labeling, and gating strategies represent as yet unsolved barriers to standardization. Nevertheless, the most widely used techniques can be summarized as follows.

ISOLATION

The main approach that has been used for isolating EVs from human fluids or culture media supernatants is a series of sequential centrifugation steps. Different purities are obtained depending on the number of steps completed. Briefly, a first centrifugation step at a low velocity (200–300 g) separates cells from EV-containing fluid, which can be further purified or directly pelleted. For a further purification, a second centrifugation must be carried out (at 2,000–10,000 × *g*, depending on the fluid or EV fraction required). Otherwise, EVs can be directly pelleted from the first supernatant (centrifuging at forces of 10,000 up to 100,000 × *g*). Though there are many variations among authors, the first approach to EV analysis is usually based on the aforementioned steps. As an alternative protocol to obtain a more pure EV fraction, a sucrose gradient can be combined with one of the centrifugation steps.

Another isolation technique is polymeric precipitation (e.g., Exoquick, System Biosciences, CA, USA). The main advantage of this approach is rapid sample processing. However, the low purity obtained and mixing of different EV subsets make results difficult to interpret.

The extraction of EVs by passing a sample through filters is a cheap and easy method that can be applied alone or combined with centrifugation. There is, however, a risk of contamination with particles other than EVs of the same size.

CHARACTERIZATION

Flow cytometry is the technique most widely employed for studying EVs (including in MS research) to the possibility of using multiple parameters to identify the same vesicle. It is a powerful characterization tool, the process is rapid and the results can be quantified. Its main limitation is poor discrimination under 0.5 μm. However, new high-resolution cytometers can detect particles as small as 0.2–0.3 μm.

Recently, two novel tools appeared on the market created to characterize nanoparticles in size and concentration with a high resolution. They measure particles based on tunable resistive pulse sensing (qNANO, IZON Science, New Zealand) and Brownian motion of the particle with nanoparticle tracking analysis software (NS500 and NS300, Nanosight, UK). The simple and user-friendly operation and powerful measurements provided by these instruments herald a new era in the analysis of EVs.

Electron microscopy is usually performed in combination with flow cytometry to provide direct evidence of the presence of EVs, and it provides what is arguably the highest quality morphological information (**Figure 1**). On the other hand, the expensive and complex processing of samples limits its use.

Fluorescence microscopy is normally used to analyze EV function *in vitro*, as well as to localize EV in tissues and budding process. In particular, confocal microscopy is widely used in EV research.

In addition, enzyme-linked immunosorbent and Western blot assays have also been employed for analysis of EVs but are less extensively used due to the poor characterization they provide and that they are difficult to quantify, respectively.

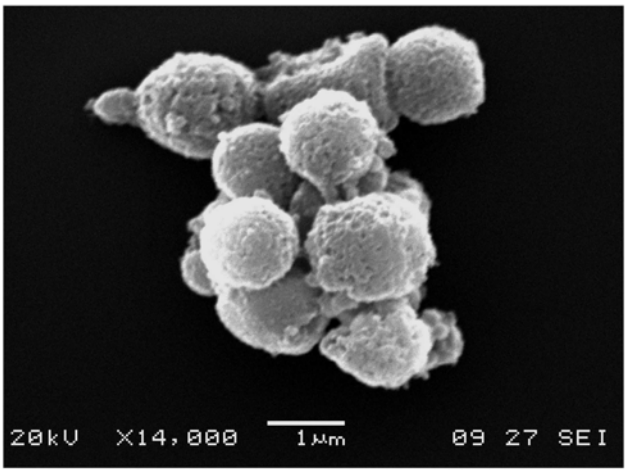

FIGURE 1 | Electron microscopy image of EVs. An electron microscopy image of an EV cluster obtained from peripheral blood. Note the rounded shape and cell membrane-like appearance of EV surfaces.

Notably, next-generation sequencing techniques are currently expanding to the field of EVs, specifically in the attempt to characterize their genetic cargo.

EVs IN NEUROSCIENCE

Vesicle secretion and the transfer of material carried within them in the CNS under physiological conditions were described many decades ago (de Robertis and Bennett, 1954). The classic example was the presence of vesicles in the neuronal synapses (de Robertis and Bennett, 1955). However, the mechanisms involved and modulation thereof by astrocytes, through the release of vesicles into the synaptic space, have only been properly understood in recent years (Antonucci et al., 2012). Vesicles have been implicated not only in the propagation of signals, but also in controlling neurogenesis with exosomes being involved in the regulation of myelin membrane biogenesis (Marzesco et al., 2005; Bakhti et al., 2011) and repairing damaged neurons (Court et al., 2011). Moreover, a recent study identified a new mechanism of regulation of the axonal integrity mediated by oligodendrocyte-derived EVs transferred to neurons (Frühbeis et al., 2013). It has been observed that EVs are released by neural cells, oligodendrocytes, neurons, microglia, astrocytes in the brain, and Schwann cells in the peripheral nervous system (reviewed by Lai and Breakefield, 2012; Frühbeis et al., 2012). All this implies that EVs perform functions necessary for growth and normal functioning of the nervous system.

In addition, EVs are involved in processes of CNS diseases carrying specific pathological cargo or performing functions that produce potential damage (Lai and Breakefield, 2012). Several studies have found variations in the number and function of circulating EVs in peripheral blood in diseases including Alzheimer's disease, dementia, epilepsy, stroke, traumatic brain injury, malaria, and tumors (mainly glioblastoma), among others (reviewed by

Lai and Breakefield, 2012; Doeuvre et al., 2009). To explore these functions, most studies expose primary cell cultures to suspensions of EVs analyzing the effects produced by EV in the cells such as morphological changes, fusion processes, induction of proliferation, and apoptosis. Another approach is to analyze EVs derived directly from human fluids. For this, peripheral blood and CSF are the most frequently studied samples. On the other hand, few studies have explored whether variations in EVs in CSF directly reflect the pathophysiology of the CNS (Morel et al., 2008; Huang et al., 2009; Street et al., 2012; Verderio et al., 2012; Mobarrez et al., 2013; Patz et al., 2013; Joshi et al., 2014) and only a couple of them have examined EVs derived from brain cells obtained from the CSF as a surrogate marker for what occurs in the CNS (Verderio et al., 2012; Joshi et al., 2014). Above all, it has not yet been elucidated whether EVs are able to migrate from the blood across the blood–brain barrier (BBB) into the CNS (or not) and vice versa (Smalheiser, 2009). More studies are required to provide evidence on whether there is an EV-mediated communication channel between the nervous and the cardiovascular systems.

MULTIPLE SCLEROSIS (MS) AS A NEUROIMMUNE DISEASE

Multiple sclerosis is a chronic autoimmune disease affecting the CNS, the cause of which remains elusive. It is, however, established that the pathogenesis of the disease involves genetic, environmental, and immune components (Bernard and Kerlero de Rosbo, 1992). There are different clinical forms, but the most prevalent is relapsing–remitting MS, characterized by outbreaks of symptoms lasting 1–3 weeks called relapses, followed by a recovery phase. During relapses, multiple areas of demyelination emerge, this being the main pathological feature of the disease. Immune activation involved in the onset of the disease causes a release of proinflammatory cytokines (TNF, IL1-beta, IFN-gamma) plus a proliferation of leukocytes, monocytes, and platelets (Martino and Hartung, 1999). At the same time, endothelial dysfunction of the BBB affects its permeability, facilitating the activation, adhesion, and transendothelial migration of monocytes and T-lymphocytes into the CNS (Minagar et al., 2012). Cytokines and chemokines released at the site of a lesion recruit glial cells, macrophages, and lymphocytes perpetuating the immune activation leading to a chronic inflammatory state (McFarland and Martin, 2007). Currently, the diagnosis of MS is based on the 2010 revised McDonald criteria (Polman et al., 2011) including careful clinical evaluation supported by MRI findings and oligoclonal banding in the CSF, the main complementary tools. The treatment of MS has undergone a revolution with the advent of IFN-beta as a treatment in the 1980s and more recently with the new immunomodulator drugs, such as natalizumab and fingolimod.

Several studies summarized in this review suggest EVs are active players in the pathophysiological development of this disease. More specifically, higher numbers of EVs have been observed in MS patients than in healthy controls and a role for EVs has been proposed in inflammatory progression and lesion repair. Because of this, they could serve as new biomarkers of disease development and targets for future treatments.

We will discuss these issues in the following sections. In **Table 1** we summarize the origins and makers used for EVs reported.

IMMUNE ROLES OF EVs IN MS

One of the necessary processes for the establishment of MS is the transendothelial migration of leukocytes into the CNS through

Table 1 | Cellular origins of extracellular vesicles (EVs) in multiple sclerosis research.

EV origin	Marker	Sample	Technique	Reference
Endothelial	CD31+/CD42−	PPP and MVEC	FC	Minagar et al. (2001)
		WB and MVEC	FC	Jy et al. (2004), Jimenez et al. (2005)
	CD51	PPP and MVEC	FC	Minagar et al. (2001)
	CD54	WB and MVEC	FC	Jy et al. (2004), Jimenez et al. (2005)
	CD106	WB and MVEC	FC	Jy et al. (2004)
	CD62E	WB and MVEC	FC	Jy et al. (2004), Jimenez et al. (2005)
	CD146	PPP	FC	Lowery-Nordberg et al. (2011)
Platelet	CD61	PFP	FC	Sáenz-Cuesta et al. (2014)
	CD41	PPP	FC	Sheremata et al. (2008)
Leukocyte	CD45	PFP	FC	Sáenz-Cuesta et al. (2014)
Monocyte	CD14	PFP	FC	Sáenz-Cuesta et al. (2014)
Astrocyte	GFAP	CSF	FM/WestB	Verderio et al. (2012)
Neuronal	SNAP-25	CSF	FM/WestB	Verderio et al. (2012)
Oligodendrocyte	MBP	CSF	FM/WestB	Verderio et al. (2012)
Microglia/macrophage	IB4	CSF	FM/FC/EM	Verderio et al. (2012)
GENERAL MARKERS				
Exosomes	CD63	PFP	WestB	Williams et al. (2013), Gatson et al. (2011)
Microvesicles	AnV	CSF	FC	Verderio et al. (2012)

EM, electron microscopy (immunogold); PPP, platelet poor plasma; PFP, platelet free plasma; WB, whole blood; WestB, Western blot; CSF, cerebrospinal fluid; FC, flow cytometry; FM, fluorescence microscopy; MVEC, microvascular endothelial cell culture.

the BBB. This migration is favored by a weakening of the barrier. The fact that this mechanism is crucial to the pathogenesis of MS is demonstrated by the benefits observed with natalizumab, which blocks the entry of leukocytes into the CNS (del Pilar Martin et al., 2008). Proinflammatory cytokines such as TNF-alpha, IFN-gamma, and IL1-beta released by inflammatory cells mediate the breaching of the BBB by the upregulation of the expression of adhesion molecules (VCAM-1, E-selectin, and PECAM-1) (Dore-Duffy et al., 1995), the loss of junctional integrity (Minagar et al., 2003), and the release of endothelial-derived EVs (EEVs) (Minagar et al., 2001). EEVs from the endothelial cells of BBB and other EVs shed from surrounding cells [leukocytes (LEV), platelets (PEV), microglia (MEV), and astrocytes] are vectors of numerous agents carried inside these vesicles or bound to their plasma membrane. The presence of metalloproteinases in EV cargo suggests that they may participate in the degradation of the extracellular matrix involved in BBB disruption (Sbai et al., 2010; Lacroix et al., 2012b). Moreover, caspase 1 carried by EVs shed by monocytes and microglia has been shown to regulate proteolytic activity of metalloproteases on endothelial cells (Bianco et al., 2005; Sarkar et al., 2009).

Minagar et al. (2001) hypothesized that plasma from MS patients contains factors that can induce endothelial activation, as suggested by the release into circulation of CD31+ EEVs from microvascular endothelial cell culture (MVEC) – a BBB model – treated with plasma from patients both in exacerbation and remission. After this pivotal study, Jy et al. (2004) demonstrated that EEVs found in plasma are able to interact and form complex with monocytes and induce their activation. These activated monocytes express Mac-1 integrin, which is an ICAM-1 receptor. The union of Mac-1 of monocytes with ICAM-1 of endothelial cells plays an important role in the transendothelial migration of inflammatory cells. Moreover, activated T cells release EVs containing the chemokine CCL5 and arachidonic acid responsible for promoting recruitment of monocytes and upregulating ICAM-1 in endothelial cells and LFA1 and Mac-1 in monocytes (Barry et al., 1998). To sum up, these data suggest that EEVs shed from the activated endothelial cells in MS patients promote the migration of monocytes and lymphocytes through the BBB and assist with the formation of demyelinating lesions. A validation of this hypothesis was performed in an elegant experiment carried by Jimenez et al. (2005): they investigated the transendothelial migration of monocytes using the MVEC model, adding plasma from remitting or relapsing MS patients and controls, and found that only the plasma from patients in relapse significantly promoted transendothelial migration. See **Figure 2** for a graphical summary of this paragraph.

Shedding new light on the role of EVs, a few recent studies have investigated EVs in the animal model of MS called experimental autoimmune encephalitis (EAE). The first published by Gatson et al. (2011) analyzed EVs (exosomes in this case) in late pregnant compared to virgin EAE mice. Results showed that EVs derived from serum of mice in late pregnancy were more numerous than those isolated from virgin mice. The proliferation of T-cells derived from splenocytes was also explored in the presence of whole serum, purified EVs, and EV-depleted serum. The three phases derived from pregnant animals were significantly more suppressive of T-cell proliferation than EVs from virgin animals or cells cultured without any EVs. On the basis of these findings, authors concluded that EVs are responsible for immune modulation during EAE pregnancy. A further study by the same group analyzed this immune modulation showing a reduction in IFN-gamma production and expression of Tbet (Th1 transcription factor) in T cells exposed to pregnancy-derived EVs. In addition, these researchers demonstrated the effect of pregnancy-derived EVs on migration to lesion areas in EAE of oligodendrocyte precursor cells and their maturation (Williams et al., 2013). This is the first publication that denoted a protective role of EVs in MS/EAE.

Verderio et al. (2012) identified other EV origins analyzing in depth CSF from humans and mice, both healthy and MS/EAE. Several types of brain cell including neurons, astrocytes, and resident microglial cells give rise to EVs. Peripheral macrophages are virtually absent in healthy brain parenchyma suggesting that myeloid EVs obtained in CSF are derived from resident microglia in the normal brain. This group also revealed that microglia store and release IL1-beta and MHC-II suggesting that the EVs produced from reactive myeloid cells may propagate neuroinflammation and provide an efficient route for rapid dissemination and presentation of antigens.

Regarding platelet-derived EVs (PEVs) in MS, P-selectin was observed on PEVs capable of binding to PSGL-1 and PECAM-1 from lymphocytes by increasing the expression of integrins such as α4β1 (VLA-4), promoting the binding of these cells to the endothelium (Sheremata et al., 2008). Interestingly, this epitope is the target of natalizumab, one of the recent therapies approved for relapsing–remitting MS.

All this evidence supports the idea that EVs are involved in MS playing a pathological role, acting as immunomodulator agents in the disruption of the BBB and the propagation of inflammation of the parenchyma but that, on the other hand, they contribute to the repair of demyelinating lesions.

ARE EVs RELIABLE BIOMARKERS IN MS?

As stated above, the association between EV concentration and the pathological condition of MS patients is clearly established. The next challenge is to develop the application of EVs as useful biomarkers: as well as providing relevant information, they are easy to process at a low cost and hence their use could be extended to large study populations. However, clearly, the adoption of EVs as biomarkers needs to be based on an objective assessment of their diagnostic and monitoring potential for the disease in question. In the case of MS, EV measurements must be correlated with the clinical judgment of the neurologist, established scores, and the results of other complementary tests such as MRI. Several studies discussed in the following paragraphs have addressed these issues but it should be noted that the results are mixed, sometimes inconsistent, depending on the type of EV (MV, MP, or exosomes), their cell origin, methods employed, and analysis performed.

EV CONCENTRATION AND CLINICAL STATUS

A relationship between EV counts in plasma and MS status was first proposed by Minagar et al. (2001) more than a decade

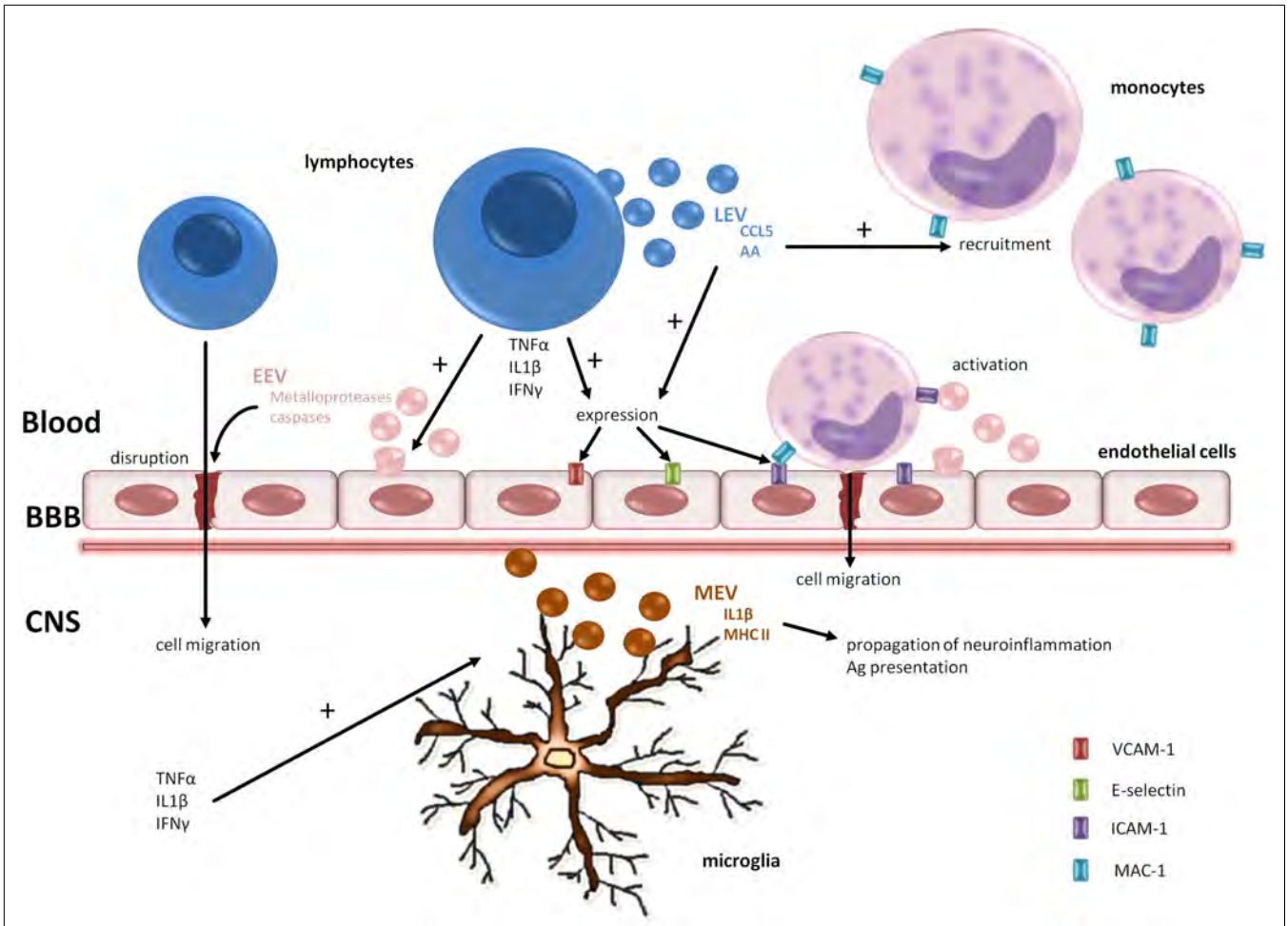

FIGURE 2 | Pathogenic roles of EV in MS. EVs are involved in the transendothelial cell migration of lymphocytes and monocytes and the spread of neuroinflammation. Metalloproteases carried by EEVs promote BBB disruption. The release of proinflammatory cytokines from lymphocytes augments adhesion molecules on endothelial cells facilitating cell adhesion. In the CNS compartment, microglia play a key role in propagation of neuroinflammation shedding MEVs containing IL1-b and MHC-II. BBB, blood–brain barrier; CNS, central nervous system; AA, arachidonic acid; EEV, endothelial-derived extracellular vesicle; LEV, leukocyte-derived extracellular vesicle; MEV, microglia-derived extracellular vesicle.

ago. Their results revealed that CD51+ EEV concentrations were higher in relapse and remission, while those of CD31+ EEVs were only higher during relapse, compared to healthy controls. They proposed that the increase in CD51+ EEVs was related to chronic inflammation owing to endothelial erosion with subendothelial matrix exposure, and that CD31+ EEVs reflect acute endothelial damage. This was tested *in vitro*, and the results were only partially reproduced. Together the findings pointed to the existence of factors present in the plasma of MS patients but not in the *in vitro* model, such as activated leukocytes present during exacerbations, which were able to regulate the release of EEVs. Authors also described a concordance between CD31+ EEV counts and gad+ MRI findings. They claimed that these vesicles were as sensitive as gad+ MRI for detecting disease activity, and also that a decrease in vesicle count could precede a negativization of MRI findings. However, this was criticized for being a premature speculation and not supported by sufficient evidence (Larkin, 2001).

In the same line of research, 3 years later Jy et al. (2004) explored whether CD54+ and CD62E+ EEVs bound leukocytes *in vitro* and in whole blood from MS patients and controls. Their main conclusion was that CD54+ EEVs form complexes with monocytes in a TNF-alpha environment and also activated them. CD62E+ EEV–monocyte complexes were more numerous during exacerbations than in remission while the number of CD54+ EEV–monocyte complexes remained unchanged, suggesting that the former would be a better marker for monitoring MS. Authors reported that the measurement of both EEV–monocyte complexes together as a single EEV–monocyte complex fraction appeared to be more sensitive to MS exacerbation than gad+ MRI and even more sensitive than the CD31+ EEV analysis studied in their previous work. Finally, free EEVs (unbound to cells) bearing CD62E allowed better discrimination of disease activity (relapsing vs. remitting patients) than CD54+ EEVs, but not compared to the previously reported CD31+ EEVs.

Conversely a year later, Jimenez et al. explored free CD54+ and CD62E+ EEVs *in vitro* reporting an increase in both markers during relapse and normal values similar to control in remission. Analyzing EEVs from relapsing patients only, CD54+ and CD62E+ phenotypes were present in significantly higher numbers than CD31+ EEVs, indicating that they were more sensitive (*in vitro*) for identifying MS status (Jimenez et al., 2005). As stated by Witwer et al. (2013), EV studies are highly heterogeneous, this being attributable to the lack of standardized methods. Possibly, this underlies the mixed results described here, particularly with respect to the earlier work by Jy and colleagues.

Besides EEVs, other EVs have been explored including those derived from platelets, leukocytes, and monocytes. Platelet activation in patients with MS may be secondary to endothelial damage (Sheremata et al., 2008). CD62P (P-selectin) levels have been shown to be higher in MS patients than controls. Twofold higher CD41+ PEV counts were found in MS patients compared to controls, these vesicles showing properties as anticoagulants (Sheremata et al., 2008). Our group also demonstrated a significant difference in CD61+ PEV, CD45+ LEV, and CD14+ MEV counts in samples from MS patients compared to those from healthy controls (Sáenz-Cuesta et al., 2014). Moreover, the PEV count was found to be higher in untreated MS patients than controls. Relapsing–remitting patients had the highest counts for the three subtypes of EVs while secondary progressive patients were found to have similar numbers to those in healthy controls. We hypothesized that EVs reflect disease status with more being shed during inflammatory periods and numbers returning to baseline during chronic progressive degeneration. Another approach to monitoring the progression of the disease is to assess patient's disability using the Expanded Disability Status Scale. Our group found no relationship, however, between EV counts and scores on this scale, and nor were the counts related to disease duration or patients' age.

In human CSF, the numbers of EVs have also been seen to be higher in patients than controls (Verderio et al., 2012). Among patients, the acute phase was associated with higher numbers of MEVs than stable or chronic phases. In addition, MEVs counts correlated linearly with gad+ MRI images. In line with this, the concentration of MEVs obtained from CSF of EAE mice reflects the course and severity of EAE. The absolute numbers of MEVs were closely associated with the course of the disease, peaking at onset and during clinical relapses, and decreasing in the chronic phase of the disease or stable phase. In this work, authors also explored the potential of MEVs as a possible biomarker in MS plotting ROC curves. Specifically, based on ROC analysis, they obtained a sensitivity of 85% and specificity of 100% for distinguishing clinically isolated syndrome patients from healthy controls, and a sensitivity of 82% and specificity of 82% for differentiating stable (relapse-free patients) from relapsing MS patients.

EVs AND MS THERAPY

Current MS therapy is based on the modulation of the immune system with a wide range of drugs. In some cases, including IFN-beta, natalizumab, and fingolimod, the effect of the drug on EVs has been explored. However, there are several new drugs, already approved (teriflunomide, alemtuzumab, BG-12) or in the final phases of testing (laquinimod, alemtuzumab, ocrelizumab), in which the potential modulation of EVs has not yet been investigated.

IFN-beta has antiviral and immunoregulatory activity mediated by its interaction with specific cell receptors on the surface of human cells. The precise mechanism of action in MS is still under investigation. So far, it is known that IFN-beta reduces the permeability of the BBB inhibiting leukocyte migration to the CNS (Calabresi et al., 1997) possibly interfering with endothelial adhesion, shifting the cytokine balance from Th1 to Th2, and increasing the expression of occludin at endothelial tight junctions (Dhib-Jalbut et al., 1996).

The effect of IFN-beta 1b on EVs was first explored by Jimenez et al. (2005) who observed an inhibitory effect on EEV production *in vitro* from MVEC culture adding plasma from MS patients, both in remission and relapse. Moreover, it was shown that monocyte–EEV complex formation and transendothelial migration are impaired after IFN-beta 1b exposure.

A first prospective study in a cohort with relapsing–remitting MS revealed a reduction in the numbers of CD31+ EEVs in plasma from week 12 of treatment with IFN-beta 1a (Sheremata et al., 2006). Conversely, no correlation was found with MRI, though there was insufficient data to draw definitive conclusions. Findings in a second cohort treated with high doses of INF-beta 1a and followed-up for a year suggest that CD54+ EEV number represents a more sensitive marker of treatment effect than CD31+ or CD146+ EEV numbers, while results showed a correlation of both CD31+ and CD54+ EEVs with T1-weighted MRI findings (the relation with CD146+ EEV failing to reach statistical significance) (Lowery-Nordberg et al., 2011). Authors speculate that the decrease they observed in plasma vesicles with IFN-beta therapy reflects a reduced interaction between CD4+ T-cells and the endothelium and subsequently less migration of the cells through a restored BBB.

Another immunomodulating drug approved (in 2006) for MS treatment is natalizumab, a recombinant humanized monoclonal antibody. A selective adhesion molecule inhibitor, binds to the alpha-4 subunit of human integrins profusely expressed on the surface of all leukocytes except neutrophils. In particular, it binds to alpha-4-beta-1 integrin, blocking the interaction with its analog receptor, the vascular cell adhesion molecule-1 (VCAM-1). Disruption of these molecular interactions prevents mononuclear leukocyte migration across the endothelium into the inflamed parenchymal tissue (Selewski et al., 2010). In a recent study analyzing plasma PEVs, LEVs, and MEVs, our group found higher counts of all three EV subtypes in IFN-beta and natalizumab-treated than untreated patients (Sáenz-Cuesta et al., 2014). No significant differences were found between the two therapies. A plausible hypothesis specifically for the rise in LEV number in natalizumab-treated patients is that blockage of leukocyte entry into the CNS would result in increase in the number of leukocytes in the blood compartment and, in turn, of LEVs in particular. The rise observed in the other two EV subtypes is, however, less well-understood.

Fingolimod is a new oral immunomodulator drug approved for the relapsing–remitting form of MS. It binds and induces

downregulation of the sphingosine 1 phosphate receptors present in lymphocytes regulating their egress from lymphoid tissues into the circulation. In that way, the drug reduces autoaggressive lymphocyte infiltration into the CNS (Chun and Hartung, 2010). Acid sphingomyelinase (aSMase) is inhibited by fingolimod (Dawson and Qin, 2011) and this enzyme controls EV production. These observations led Verderio et al. to theorize that using fingolimod could inhibit MEV shedding from reactive microglia and also macrophage infiltration into the CNS. Their experiments in an EAE model confirmed that MEV numbers decreased to baseline levels in the CSF with the administration of fingolimod. In mice, symptom scores and MEV counts were correlated during fingolimod treatment. Hence, a novel effect of fingolimod was postulated, namely, that it limits the spreading of the inflammatory signal by impairment of MEV production (Verderio et al., 2012). Despite these conclusions, there have so far been no reports evaluating the effect of fingolimod on EVs in humans, probably because it has only relatively recently become available commercially.

Apart from being biomarker for treatment response, it has been proposed that engineered EVs be loaded and used to deliver exogenous compounds for therapeutic purposes, raising the prospect of a novel clinical application for EVs. Preliminary studies with exosomes have been carried out in some types of cancer (Kosaka et al., 2013); however, more research is required before this approach can be used in clinical practice as a complementary therapy. Particularly in MS, a recently published study explored the ability of exosomes packed with microRNA to increase baseline myelination, reduce oxidative stress, and improve remyelination (Pusic et al., 2014). The results showed a significant increase in myelination in hippocampal slice cultures. Nevertheless, the effects of this therapeutic approach need to be investigated further, first in an animal model such as EAE and later in a clinical trial.

CONCLUDING REMARKS

Extracellular vesicles play important roles in the development of MS, in particular activating cells during relapses, leading to migration through the BBB, and spreading inflammation in CNS tissue. On the other hand, a protective effect of EVs has been described with the induction of maturation and migration of oligodendrocyte precursor cells.

Regarding the application of EV research findings to daily clinical practice, it is not yet possible to propose EVs as a specific biomarker for MS due to no compounds having been sufficiently closely linked to the disease. Nevertheless, there is evidence that they reflect disease progression. Particularly, EEVs, PEVs, and MEVs appear to be the most accurate markers. What is more, the effects of treatments seem to be reflected in EV counts. We consider it likely that new carefully designed studies with longer follow-up periods will allow us to confirm the involvement of EVs suggested by our current knowledge and open future applications.

Finally, there is an urgent need for consensus guided by the new scientific societies for EVs to standardize the methodologies and instruments used, in the analysis of EVs with potential applications in clinical practice, and thereby make it possible to obtain comparable results.

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Methods for extracellular vesicles isolation in a hospital setting

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The research in extracellular vesicles (EVs) has been rising during the last decade. However, there is no clear consensus on the most accurate protocol to isolate and analyze them. Besides, most of the current protocols are difficult to implement in a hospital setting due to being very time-consuming or to requirements of specific infrastructure. Thus, our aim is to compare five different protocols (comprising two different medium-speed differential centrifugation protocols; commercially polymeric precipitation – exoquick – acid precipitation; and ultracentrifugation) for blood and urine samples to determine the most suitable one for the isolation of EVs. Nanoparticle tracking analysis, flow cytometry, western blot (WB), electronic microscopy, and spectrophotometry were used to characterize basic aspects of EVs such as concentration, size distribution, cell-origin and transmembrane markers, and RNA concentration. The highest EV concentrations were obtained using the exoquick protocol, followed by both differential centrifugation protocols, while the ultracentrifugation and acid-precipitation protocols yielded considerably lower EV concentrations. The five protocols isolated EVs of similar characteristics regarding markers and RNA concentration; however, standard protocol recovered only small EVs. EV isolated with exoquick presented difficult to be analyzed with WB. The RNA concentrations obtained from urine-derived EVs were similar to those obtained from blood-derived ones, despite the urine EV concentration being 10–20 times lower. We consider that a medium-speed differential centrifugation could be suitable to be applied in a hospital setting as it requires the simplest infrastructure and recovers higher concentration of EV than standard protocol. A workflow from sampling to characterization of EVs is proposed.

Keywords: extracellular vesicles, protocol standardization, clinical application, nanoparticle tracking analysis, flow cytometry, translational research, urine

INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound particles shed from almost all cell types, carrying components from the cell donor such as lipids, proteins, RNA, glycolipids, and metabolites (1). It has been suggested that they play several biological roles like, for example, antigen presentation without cell contact (2), microenvironment modification, and distant cell education (3), roles that have been encompassed under the term "cell-to-cell contact-free communication". In turn, their biological functions have been related to many pathophysiological processes, the most studied being cancer (4), immune-mediated diseases (5), and cardiovascular disorders (6).

A widespread concern in the biomedical research community is the gap between the basic research carried out in the laboratories and the clinical setting where the new biological information should have a direct impact. Many researchers have directed their efforts toward bridging that gap and look for ways to translate lab findings into clinical solutions, emerging therefore the translational research. The translational research on EVs is not foreign to this goal: the current knowledge about EVs, mostly developed *in vitro*, has been proposed to be applied in a daily hospital routine giving answers to specific health queries (7–13). This possible application ranges from diagnostic to therapeutic objectives, including disease monitoring and the search of prognostic biomarkers, among others. But are the hospitals technologically prepared to employ EVs studies routinely?

The main steps for studying EVs and applying the results involve sampling (blood, urine, saliva, cerebrospinal fluid, joint

fluid, breast milk, ascitic fluid, etc.) and isolation, to be afterward characterized and analyzed their cargo and, finally, give a potential clinical interpretation and application. Concerning the first steps, sampling and pre-analytical parameters have been widely studied and are close to reach a consensus (14, 15). However, isolation is still a critical step due to several reasons. First, the methods to isolate EVs are currently highly diverse [reviewed by Momen-Heravi et al. (16) and Witwer et al. (17)] and depending on which one is employed, the results can be considerably different, even having started from the same sample. At the moment, most of them are based on EV density, including differential centrifugation steps from low speeds (1,500 × *g*) to ultracentrifugation (>100,000 × *g*), combined or not with density gradient and/or filtration. Precipitation using polymers and immunoaffinity agglutination are also widely used. Recently, the size-exclusion chromatography (18) and chip devices (19) have been added to the rest of methods. All of them, either by themselves or in combination, yield a solution enriched in EVs in different extents. Finally, depending on several factors such as time consumption, cost, friendly use, and reproducibility, these methods are or not able to be applied in a daily clinical routine. Despite lots of important works shedding light on this field, there is still a lack of consensus (20) evidencing the urgent need of standardized protocols appropriate for hospitals.

Considering the problems and needs regarding the use of EVs in a clinical setting, we established the following objectives for the present study: to compare several protocols for EVs isolation and to analyze which of them could be the most suitable one to be used in daily clinical setting.

MATERIAL AND METHODS

Blood and urine are the most widely used samples in a hospital setting, as they provide useful information and they are easy to obtain with minimally invasive techniques. Thus, we decided to isolate EVs from these biofluids as the starting point for EV isolation.

Samples were collected from 10 healthy individuals (5 males and 5 females; average age = 37 ± 8 years old) and stored according to the criteria of the Donostia node of the Basque Biobank. All subjects gave written informed consent and the study was approved by the Hospital Ethics Committee.

Donors underwent a questionnaire about recent exercising (within the last hour), drugs/medication intake, ovulatory cycle, acute illness, and sleeping hours.

The workflow followed in the present work is summarized in **Figure 1**.

BLOOD

Peripheral blood samples were collected at the Donostia University Hospital at 8:30 a.m. on fasting and were processed separately (without pooling them) within the first hour. After discarding the first milliliter, blood collection was done by venipuncture with a

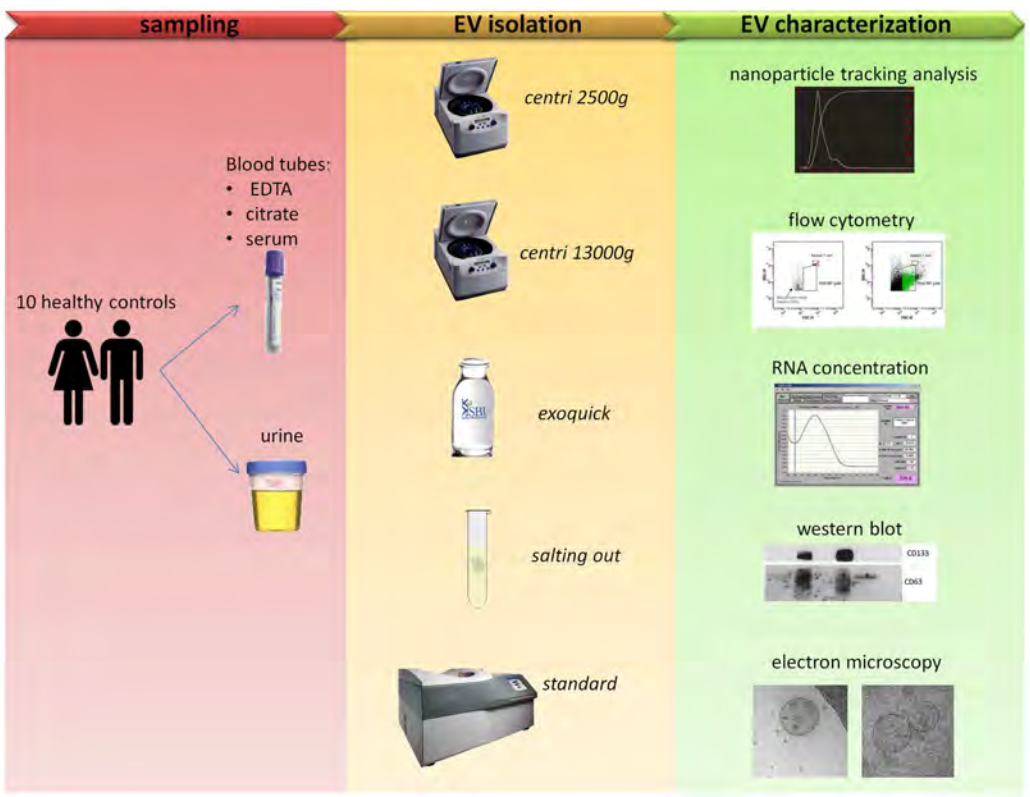

FIGURE 1 | Comparison of five protocols for EVs isolation. Blood and urine samples were isolated with five different protocols (see **Table 1** for more details) and then characterized with five outputs methods.

21-gage needle in a 10-ml EDTA tube, a 3.8-ml citrate tube, and a 8-ml serum tube [Vacutainer, Becton Dickinson (BD)], kept upright and centrifuged at 2,500 × *g* during 15 min. The supernatant was recovered to obtain platelet-poor plasma (PPP) or platelet-poor serum, being these samples the starting point for all the protocols. Besides, additional blood samples were collected in EDTA and serum tubes to perform a hemogram and obtain protein and lipid profiles in the core laboratory of the Hospital. The parameters tested are shown in **Table 1**.

Although we collected plasma and serum from peripheral blood, the present work is focused on plasma. In this sense, we are going to refer only to plasma in all the sections of "Materials and Methods" for the sake of simplicity. The results and discussion of blood-derived EVs will be also centered on plasma and only relevant results will be presented in the case of serum.

URINE

Sixty milliliter-first void of the day was collected in aseptic conditions by each individual at home, kept at 4°C until their processing with an average time of 2.7 h between collection and processing. Ten milliliters were sent to the core laboratory for the analysis of the most common urine parameters (**Table 1**). The rest was aliquoted in five tubes of 10 ml and centrifuged at 2,500 × *g* for 15 min in order to obtain cell-free urine (CFU).

EV isolation protocols

The protocols described below are summarized in **Table 2**.

Centri2500. This method is based on the protocol published by Lacroix and colleagues (14). Briefly, 1.3 ml of PPP or 9.5 ml of CFU obtained at the first centrifugation are centrifuged again at 2,500 × *g* during 15 min to get 1 ml of platelet-free plasma (PFP) or 9 ml of debris-free urine (DFU). Both PFP and DFU samples were stored at −80°C for later use. When needed, samples were thawed on ice and centrifuged once again at 20,000 × *g* during 20 min to pellet the EVs, discarding 900 μl of supernatant, following the protocols described by Ashcroft and colleagues (21) and Jayachandran and colleagues (22). The pellet containing the EVs was resuspended in 100 μl of PBS (GIBCO, Life Technologies) filtered twice through a 0.22 μm-pore filter.

Centri13000. This method is based on the protocol published by Dey-Hazra and colleagues (23) and Dignat-George and colleagues (24). In brief, it is a modification of the previous method where the second centrifugation performed on the 1.3 ml of PPP was done at 13,000 during 2 min to obtain PFP or DFU. The rest of the protocol was the same as the previous one. To note, this protocol was not performed for urine samples.

Exoquick. The basis for this method lays on the precipitation of EVs using a commercial agglutinating agent and was performed following the manufacturer's instructions. In summary, 63 μl or 2 ml of exoquick TC (System Biosciences) were added either to 250 μl of PPP or to 9.5 ml of CFU, respectively, and the mix was incubated overnight at 4°C with no rotation. Then, two centrifugation steps were performed at 1,500 × *g* for 30 and 5 min, respectively, to sediment the EVs and the pellet was resuspended in 200 μl of PBS. It needs to be noted that, although the first versions of the manufacturer's instructions included a filtering step using a 0.45 μm-pore filter, it was removed in the latest version and, thus, it has not been included in our protocol.

Salting out. This method has been adapted from the protocol recently published by Brownlee and colleagues (25) and it is based on the precipitation of EVs through an aggregate of sodium acetate 1 M, ph 4.75. A centrifugation was performed on 1.3 ml of PPP or 9.5 ml of DFU at 13,000 × *g* for 30 min; we collected the supernatant (1 ml of PPP or 9 ml of DFU), added sodium acetate (dilution 1/10), and incubated on ice for 60 min and, subsequently at 37°C for 5 min. The dilution was then centrifuged at 5,000 × *g* during 10 min and the resulting pellet was washed with

Table 1 | Lab parameters analyzed in blood and urine samples.

Blood	Creatinine (mg/dl)	0.9 ± 0.3
	Total cholesterol (mg/dl)	184.9 ± 37.7
	HDL (mg/dl)	69.9 ± 14.1
	Triglycerides (mg/dl)	65.7 ± 20.1
	LDL (mg/dl)	101.9 ± 37.5
	Total proteins (g/dl)	7.2 ± 0.6
	Albumin (g/dl)	4.3 ± 0.3
	Hematocrit (%)	41.7 ± 3.4
	Leukocyte (10e3/μl)	7.0 ± 2.5
	Platelet count (10e3/μl)	238.7 ± 56.7
	Lymphocyte count (10e3/μl)	1.9 ± 0.5
Urine	Density (g/l)	1019.4 ± 7.7
	pH	6.0 ± 0.9
	Glomerular filtrate (mL/min/1.73 m^2)	84.3 ± 15.5
	Erythrocyte count (ery./μl)	Negative
	Leukocyte count (leu./μl)	Negative
	Epithelial cell count (cells/μl)	Negative

Values represent the mean ± SD from the 10 healthy controls.

Table 2 | EV isolation methods compared in this work.

Method	Isolation principle	Steps
Centri2500	Differential centrifugation	2500 × *g* 15′ × 2 + 20,000 × *g* 20′ to pellet the EVs
Centri13000	Differential centrifugation	2500 × *g* 15′ + 13,000 × *g* 2′ + 20,000 × *g* 20′ to pellet the EVs
Exoquick	Agglutination–precipitation	2500 × *g* 15′ + agglutination with exoquick + 1500 × *g* 30′ and 5′ to pellet the EVs
Salting out	Precipitation	2500 × *g* 15′ + 13000 × *g* 30′ + acid precipitation + 5000 × *g* 10′ to pellet the EVs
Standard	Differential centrifugation – size filtration – ultracentrifugation	2500 × *g* 15′ + 0.22-μm filter + 10,000 × *g* 30′ + 1,00,000 × *g* 75′ to pellet the EVs

a buffer with sodium acetate at 0.1 M to be finally resuspended in 200 μl of PBS.

Standard. This is the method considered as the standard isolation protocol nowadays (26). The starting point was 1.3 ml of PPP or 9.5 ml of CFU that were filtered through a 0.22 μm-pore filter and centrifuged at 10,000 × *g* during 30 min to obtain either PFP or DFU. These were ultracentrifuged at 100,000 × *g* in an Optima MAX tabletop centrifuge (Beckman Coulter) during 75 min. The resulting EV pellet was resuspended in 200 μl of filtered PBS.

EV detection and characterization methods

Nanoparticle tracking analysis. The size distribution and concentration of EVs were measured using a NanoSight LM10 machine (NanoSight). All the parameters of the analysis were set at the same values for all samples and 1 min-long videos were recorded in all cases. Background was measured by testing filtered PBS, which revealed no signal. The EVs obtained from PFP (5 μl) were diluted with filtered PBS to 1:150 and the ones obtained from DFU (5 μl) to 1:50. For each sample, two measurements were performed. It is necessary for a minimum of 200 tracks (movements of single particles recorded by a camera) to obtain valid results. The following parameters were measured: the mean and mode of the size distribution and the concentration of EVs (27).

Flow cytometry. The labeling and gating of EVs were performed as described by Sáenz-Cuesta and colleagues (28). Briefly, 4 μl of CD61-PE (Cytgonos) or CD45-PE (BD) monoclonal antibodies were mixed with 40 μl of resuspended EVs and incubated for 20 min. Next, labeled EVs were washed once with 300 μl of filtered PBS, resuspended in further 200 μl of filtered PBS and acquired at low rate in a FACS Canto II flow cytometer (BD). Side and forward scatter were measured on a logarithmic scale with the threshold set at 300 for each parameter to avoid instrument noise (background signal). Then, the lower limit was defined with the exclusion of background noise given by the signal of PBS filtered twice. To define the upper limit of the total MP gate, 1-μm non-labeled polystyrene latex beads were used (Sigma-Aldrich). The events that appeared in this region were included in the total EV count and were further analyzed for specific labeling (positive for PE marker). We defined CD61+ EVs as platelet-derived EVs (PEV) and CD45+ EVs as leukocyte-derived EVs (LEV). The total and cellular origin-specific EV concentrations were obtained using Trucount™ tubes [BD; Ref. (28)].

Western blot. Primary CD133 (Miltenyi Biotec S. L) and CD63 (Santa Cruz Biotechnology, Inc.) antibodies were used to study specific EV transmembrane markers. Mouse and rabbit HRP-conjugated antibodies (Cell Signaling) were employed as secondary antibodies. All protein procedures were done at non-reducing conditions. Samples (10 μl of PBS-resuspended EVs) were incubated at 95°C for 5 min, separated in SDS polyacrilamide gels, and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked for 1 h at room temperature with 5% milk (w/v) in TBS solution with 0.1% Tween-20 (T-TBS) and incubated in the same solution with primary antibodies overnight at 4°C. Primary antibodies were washed with the T-TBS solution and incubation with secondary HRP-conjugated antibodies was performed at room temperature for 1 h in the same solution used for the primary antibodies. After washing with T-TBS solution, the HRP signal was detected by a chemiluminiscent reaction with the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Inc.).

RNA isolation. A 185 μl-aliquot of resuspended EVs was used to extract total RNA with the miRNeasy serum/plasma kit (Qiagen). RNA concentration was measured with the nanodrop 1000 spectrophotometer (Thermo Scientific).

Cryo-electron microscopy. The cryo-electron microscopy (EM) was performed following the protocol used by Perez and colleagues (29). Briefly, 10 μl of EV preparations were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL Micro Tools GmbH). Grids were blotted at 95% of humidity and rapidly plunged into liquid ethane with the aid of VITROBOT (Maastricht Instruments B). Vitrified samples were imaged at liquid nitrogen temperature using a JEM-2200FS/CR transmission cryo-electron microscope (JEOL) equipped with a field emission gun and operated at an acceleration voltage of 200 kV.

Statistical analysis

The statistical analysis was performed with PASW Statistics v18.0 (SPSS Inc.). Kolmogorov–Smirnov and Shapiro–Wilk tests were used to check normality of distributions. As all of the variables were shown to follow a normal distribution, *T*-tests were applied to assess differences between the groups. Pearson's *R* correlations were computed to explore the relations between lab parameters and some EV parameters. Both differences between groups and correlations between variables were considered significant when $p < 0.05$.

RESULTS

BLOOD

EV concentration

Extracellular vesicles concentration was measured using two independent methods: nanoparticle tracking analysis (NTA) and conventional flow cytometry (FC). It is to be noted that the lower detection limits are different, being 50 nm for NTA (27) and around 400 nm for FC (30).

Nanoparticle tracking analysis

When using NTA, the software requires a minimum of 200 tracks during the capture time of the video. In the case of the samples processed with the salting out and standard methods, only few of them reached that minimum. This was critical for NTA analysis causing a high variability on these samples (**Figures 2A,E**). The exoquick method yielded higher EV concentration values than any other method used. We obtained four times higher EV concentration with exoquick than with centri2500 ($p = 0.007$) and centri13000 ($p = 0.05$) and 23 times higher concentration values comparing to salting out ($p = 0.002$) and standard ($p = 0.002$) methods (**Figure 2A**). No significant differences have been found either between the EV concentrations obtained with the centri2500 and centri13000 methods, or between those yielded by the standard and the salting out methods. However, there are significant

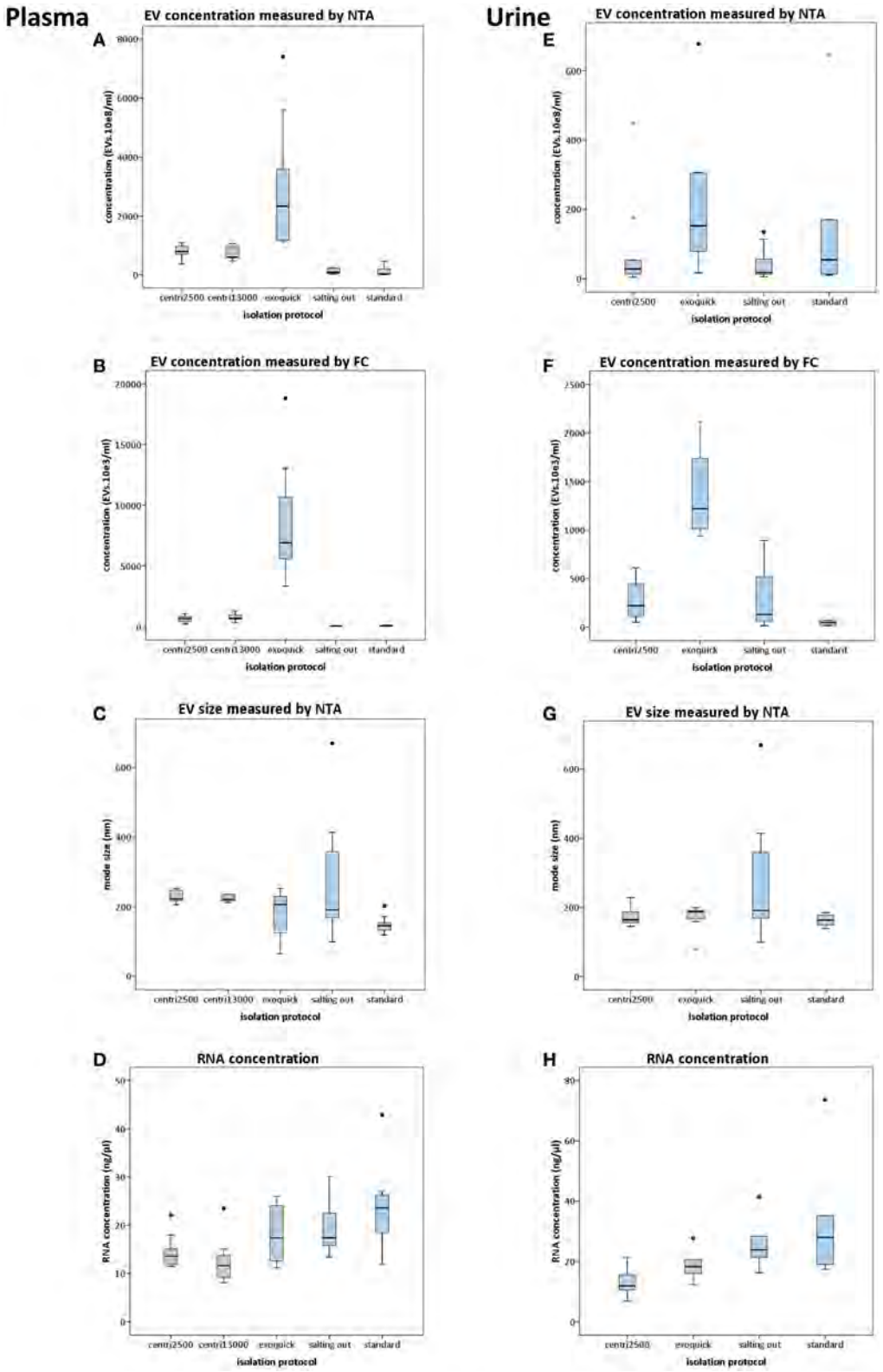

FIGURE 2 | Results of the comparison of five protocols for isolation of EVs. Box plots show EV concentration measured by nanoparticle tracking analysis [NTA; **(A,E)**] or conventional flow cytometry [FC; **(B,F)**], EV size distribution measured by NTA **(C,G)**, and concentration of RNA yielded from EVs **(D,H)**. In the left column, the results from plasma-derived EVs are shown and in the right column those from urine. For statistical significance, see text. All bars represent mean values with SD except for size plots **(C,G)** that bars indicate mode with SD.

differences between these two groups of methods (**Figure 2A**) obtaining a p value of <0.001 for both centri2500 vs. salting out and standard and 0.002 and <0.001 for centri13000 vs. salting out and standard respectively. Regarding the EVs isolated from serum, they were obtained using the exoquick, centri13000, and standard protocols and we have observed that, as it happens with plasma, the exoquick method yields significantly higher EV concentrations than the other two methods. When we compared serum and plasma considering all isolation methods, 3.4 times higher EV concentrations have been observed for serum using exoquick and 1.3 times higher ones when using centri13000, but these comparison did not reach statistical significance (see Table S1 in Supplementary Material).

Flow cytometry. Although the EV concentrations obtained using FC were lower than those obtained by NTA, there was good correlation between the concentration profiles yielded by each approach when using the averages for each isolation method for the comparison ($R = 0.99$; $p < 0.001$). Nevertheless, no significant correlations were observed when performing the analysis for each isolation method separately. Besides, significant differences were observed between all isolation methods except for *centri2500* vs. *centri13000* and *salting out* vs. *standard* (**Figure 2B**).

EV size

There is great similarity between the modes of EV size obtained with the different isolation protocols ranging from 150 to 277 nm, average of mode size: 228 nm (**Figure 2C**). In accordance to these results, the EM images show EVs with a size between 100 and 200 nm (**Figure 3A**). Significant differences in size only exist between the EVs isolated with the *standard* method when compared to those obtained through the *centri2500* and *centri13000* methods, being the former the smallest of all at 158.7 nm. Besides, the EM images of the EVs obtained with *exoquick* present several filamentous aggregates and other globular structures not considered EVs.

RNA concentration in EVs

Despite the *salting out* and the *standard* methods being the ones that yield the lowest EV concentrations, the highest RNA concentrations have been obtained through these methods (19.5 ± 5.7 and 23.6 ± 8.2 ng/μl, respectively), higher than with the *centri2500* and *centri13000* methods (14.5 ± 3.3 and 12.5 ± 4.5 ng/μl, respectively). It is remarkable that *exoquick* yields 18.1 ± 6.0 ng/μl of RNA, despite having isolated around 23 times more EVs than the *salting out* and *standard* methods (**Figure 2D**).

Western blot

The detection of EV markers through western blot (WB) was used as a confirmation of the presence of EVs in the solutions obtained at the end of the isolation protocols. The objective was not to perform a detailed characterization of the markers, but to look for differences in their detection between the different methods.

Great inter-method variability has been observed. Briefly, CD63 detection is better in EVs isolated from plasma than in those obtained from serum. Among the plasma-derived EVs, CD133 showed better signal for *centri2500* and *centri13000* than for the *salting out* and the *standard* protocols. The detection of CD133 was also worse in serum-derived sample than in plasma-derived samples. Nonetheless, CD133 detection was better in urine-derived EVs than in the samples obtained from blood (**Figure 3B**). It is of note that the EVs isolated with *exoquick* could not be used for WB marker analysis using plasma samples due to be impossible to dissolve its pellet.

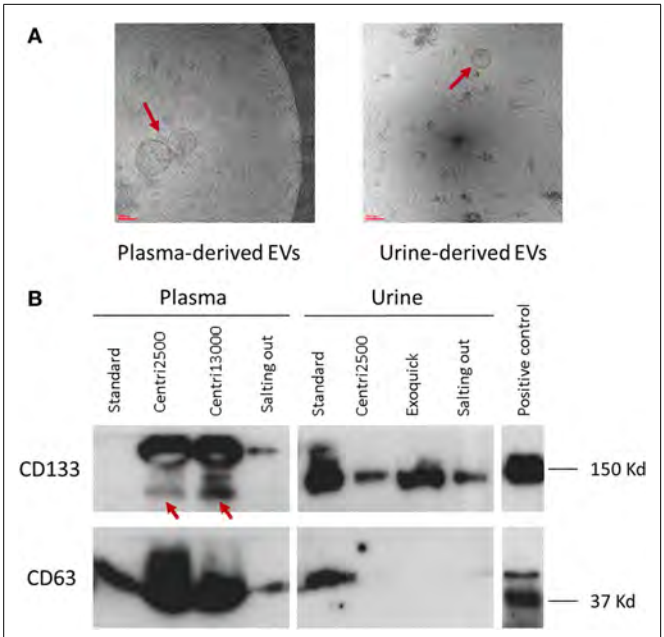

FIGURE 3 | Characterization of EVs. **(A)** Two images of electronic microscopy of EVs (pointed with red arrows) derived from plasma and urine. Scale bar represents 100 nm. To note, in the image of urine-derived EVs, it is observed contaminants not seen in the plasma-derived one. **(B)** Western blot analysis using the specific EV markers CD133 and CD63. In CD133 plasma samples, the specific bands are pointed with red arrows. Positive control was performed with NHC-2 p10 cell line.

URINE

EV concentration

In the NTA analysis of the EVs isolated from urine, we have observed that the concentrations were as low as the great majority of the samples have not reached the minimum track-count. Thus, once again the interpretation of NTA data from urine-derived EVs was carried out with caution. *Exoquick* was the method that yields the highest EV concentrations from urine using either NTA or FC, followed by the *standard* protocol. The *centri2500* and *salting out* protocols yielded very similar concentrations. **Figures 2E,F** summarizes the EV concentration results from the application of the different protocols to urine as measured by NTA and FC, respectively (for p values, please see Table S1 in Supplementary Material). In summary, using both NTA and FC, we obtained a similar concentration profile to that of blood but with 10–20 times lower concentrations.

EVs size

The modes of the size distributions obtained with the different protocols were similar and, the average of these modes (207 nm)

was similar to that achieved in blood samples (228 nm). As it happened with blood samples, the smaller EVs were isolated with the *standard* method (162.5 nm) comparing to the other protocols (**Figure 2G**). The results of the size assessed by NTA were, once again, consistent with the observations done using EM. In most of these urine samples, the number of contaminating particles that could not be considered EVs was higher than in blood (**Figure 3A**).

RNA concentration in EVs

Higher RNA concentrations were obtained from EVs isolated with the *standard* (33 ng/μl) and *salting out* (25.9 ng/μl) methods and even higher than the concentrations obtained from blood samples, where the EV concentration was from 10 to 20 times higher than in urine (**Figure 2H**).

Western blot

The EV samples isolated from urine using *exoquick*, in contrast to the ones isolated from plasma, can be used for marker analysis with WB (**Figure 3B**).

In brief, CD133 detection in urine-derived samples is better with precipitant agents (*exoquick* and *salting out* methods) than with centrifugation methods, although the *standard* shows better results in urine and serum than in plasma. On the opposite, CD63 signal is weak in urine-derived samples compared to that of plasma- or serum-derived samples for all methods.

CORRELATION WITH LABORATORY PARAMETERS

We have also tested whether the EV concentration values obtained with different methods and the different types of samples (plasma, serum, or urine) could be reflected in some of the lab parameters measured in blood and urine, especially the ones that are related to the main components of EVs, i.e., lipids and proteins. Furthermore, the analysis of these correlations would serve to test a possible interference in EV quantification produced by these parameters, a phenomenon that has been previously described (31).

Interestingly, a significant correlation has been observed between the concentration, as measured by NTA, of the EVs isolated from plasma with the *centri13000* method and the total cholesterol ($R = 0.953$; $p = 0.003$) and LDL concentrations ($R = 0.935$; $p = 0.006$) in blood. We have also detected a significant correlation between the NTA-measured concentration of the plasma-derived EVs isolated using the *standard* method and the concentration of triglycerides in blood ($R = 0.789$; $p = 0.007$).

No significant correlation has been found between the concentrations of the EVs of specific cell-origins and the concentrations of the respective source cells in blood. The concentrations of CD61+ (platelet origin) or CD45+ (leukocyte origin) EVs are plotted in Figure S1 in Supplementary Material.

In regard to the EVs isolated from urine, the only significant correlation we have observed is the one between the density of urine and concentration of EVs, measured with both NTA and FC, isolated with the *salting out* protocol ($R = 0.841$; $p = 0.002$ for NTA and $R = 1.000$; $p = > 0.001$ for FC).

DISCUSSION

In the present work, we have studied and compared several widely used methods for the isolation of EVs, including differential centrifugation, agglutination, precipitation, and the one considered the standard that includes ultracentrifugation (plus filter). All methods under study can be applied using relatively simple technology, with the exception of ultracentrifugation, which must be performed with an instrumentation that, even if it is easy to use, is not usually found in most hospital laboratories. The election of one or other method as the most suitable one to be used in a hospital setting greatly depends on the goals to be reached with the method, which could be, among others: to maximize the final EV concentration, to obtain high levels of purity as measured by markers and several classical characteristics of EVs, to select one of the three fundamental types of EVs (exosomes, microvesicles, and apoptotic bodies) or to get the less time and/or money consuming protocol. We have set the first two as preferential aims, leaving the rest out of the scope of this work.

ISOLATING EVs

We have observed that, besides being the method that can be implemented most easily (it is quick and relies on very little technology), *exoquick* is also the method that yields, in a statistically significant manner, the highest concentration of EVs (as measured by NTA and FC) compared to the other four isolation protocols. The EV quantity is even higher when using serum as the starting sample. On top of that, to dissolve the pellet obtained using *exoquick* from serum-derived samples is notably easier than plasma-derived samples. Nonetheless, the considerably higher EV concentrations obtained with *exoquick* (23 times higher than those obtained with the *standard* protocol) could be linked to the aggregation and precipitation of other elements in suspension in the sample that are not necessarily EVs; as it can be observed in the images obtained by EM. Taylor and colleagues (32) demonstrated that using *exoquick* more EVs are isolated than using ultracentrifugation (*standard*), chromatography, and magnetic beads, and with a higher purity of exosomal RNA and proteins. Our results only partially coincide with the observations of Taylor and colleagues, as the RNA concentration we obtained with *exoquick* is lower than that yielded by the *standard* method. In another study that compared the *exoquick* method with the *standard* method, the authors concluded that a combination of these two methods is the protocol that yields the highest EV counts, although exosomes of higher quality were obtained combining the *standard* method with the sucrose density gradient (33). Yet, *Exoquick* is the most expensive of the methods used in the present work.

The differences in EV concentration between the two centrifugation methods (*centri2500* and *centri13000*) are not statistically significant. The sole differences are that the cluster of EVs observed by FC in the FSC/SSC dotplot shows less debris around in the case of *centri13000* and that the EVs isolated with this method also show a stronger labeling of CD63 in WB. The conclusions reached at the workshop of the Scientific and Standardization Committee of the International Society of Thrombosis and Hemostasis to promote the use of these protocols (15) and aimed to reducing the variability due to a resuspension of the pellet (24). Nonetheless, in our opinion, the main drawback of this proposal is that, as the EVs are not concentrated in a pellet–like, we performed with the final centrifugation at 20,000 × *g*, a pellet-washing step cannot be introduced and EVs are maintained in dissolution along with many other contaminating particles such as protein aggregates.

Regarding size, very similar EV sizes have been obtained with these two methods, even when measuring size on EM imagery. These suggest that the second centrifugation is probably not that critical and could vary, at least between 2,500 and 13,000 × *g*, with the objective of eliminating cell debris. Moreover, these methods collect six times higher EV concentrations than the *standard* protocols, what can be explained by the fact that they are less restrictive methods. Finally, the technical requirements for the use of these methods are usually met in most basic research laboratories and they are considerably less time-consuming than the *standard* method.

Ultracentrifugation is nowadays the "*gold standard*" method for the isolation of EVs, fundamentally exosomes. With the aim of finding alternative methods to this protocol, Brownlee and colleagues (25) have recently described a new method called *salting out*, based on the precipitation of EVs using the aggregate of acetic acid. In the present study, the *salting out* method yielded the lowest EV concentrations when compared to the other protocols, although showing similar values to those obtained with the *standard* method as the authors of the aforementioned article also pointed. It has to be noted, though, that Brownlee and colleagues isolated EVs from cell culture supernatants and, thus, comparisons with the present work must be done with caution.

Although out of the main objectives of this work, we have also compared the EVs isolated from three different types of samples: plasma, serum, and urine. We have observed that higher concentrations of EVs are obtained from serum than from plasma for all methods, and 10–20 times more, depending on the method, when comparing plasma with urine. As comparisons between serum and plasma have been performed by other authors (34, 35), we just present our results.

The EV size distributions that we have obtained with the *exoquick*, *salting out*, *centri2500,* and *centri13000* methods are very similar, being the EVs with a size below 200 nm the most abundant. Nevertheless, a cluster of EVs can be observed with a size around 500–600 nm that could represent the population of microparticles. On the contrary, the standard method isolates smaller EVs as it uses a 0.22 μm-pore filter leaving out the bigger EVs (microparticles and apoptotic bodies). We agree with Jy and colleagues (36) that the capacity of the first four methods to isolate the bigger EVs can be useful when applying these protocols in clinical practice.

In the case of urine, very low EV concentrations have been obtained with the five methods and, thus, we recommend not to dilute or to dilute very little urine-derived samples before analyzing them by NTA, FC, and WB. Once again, *exoquick* was the method that yields the highest concentrations according to other authors' results (37). Certainly, when using urine samples, it would be of great consequence to avoid contaminating proteins such as Tamm–Horsfall, which traps EVs but it can be removed with the simple addition of dithiothreitol and heat (38). Furthermore, Rood et al. (18) suggest that the most effective method in terms of purity for urine-derived EVs to undergo downstream proteomic analysis is the combination of ultracentrifugation followed by size-exclusion chromatography. The major disadvantage of this protocol would be that it is time-consuming and it requires of specific infrastructure that make it difficult to be compatible with clinical applications.

DETECTING AND CHARACTERIZING EVs

During the processing, after the centrifugation at 20,000 × *g* for 20 min, a fine lipidic layer could be observed in some of the samples. This corresponded to a FC image with a higher EV density (data not shown). Nevertheless, the presence of this layer did not show correlation with NTA results. It is well known that the density and size of the EVs can overlap with these of lipoproteins and this can produce artifactual results in FC analyses (31). Besides, we have found positive correlation between the LDL levels in blood and the concentration of EVs obtained with several methods, which suggests that, when isolating the EVs, some LDL particles are also dragged and counted as EVs. One approach to measure the purity of EVs is the EV/protein ratio (39), a method that is easy to use and yield reproducible results. However, it remains out of the scope of the present work.

The most widely used methods for the quantification of EVs are NTA and FC. According to our data, the results yielded by these two methods are not interchangeable, probably because the size ranges that they can analyze are different. The correlation between the two methods would be better studied using only the concentration of EVs larger than 400 nm, as this is the minimum size for the FC analysis. Nevertheless, we have looked for correlation using concentration results for EVs larger than 400 nm in another dataset (data not shown) and we have found none. Thus, we consider that these two quantification methods do not exclude each other but are complementary, as NTA gives more accurate counts but FC allows the characterization of distinct cellular origins.

From the comparison of the methods that we have used to study the size of EVs, we can conclude that, while the NTA, as it allows to recover information from individual particles, allows to obtain and compare size distributions, EM provides more robust information on the characteristics of EVs but size distributions cannot be obtained through EM imagery. Furthermore, NTA has the advantage of performing a multiple analysis in few minutes.

Tetraspanins have been widely used as general markers of EVs; however, during the last years, some works have provided evidence that not all vesicles express them at the same levels suggesting that different EV subsets could coexist in the same pellet (40, 41). In the case of urine-derived EVs, our results present low or undetectable levels of CD63 except for those obtained with the *standard* protocol (**Figure 3B**). Both the previously described lack of CD63 in urine-derived EVs larger than 100 nm (42) and its expression in EVs obtained with the *standard* protocol (43, 44) are congruent with our results. In the other hand, we found expression of CD133 with all the methods. In agreement with other authors, we concluded that the presence of CD133+/CD63− EVs demonstrate the recovery of the large ones that usually express this pattern of markers (45–47). Moreover, Bobrie and colleagues described the CD63 as a variable marker found only in a fraction of the sucrose gradient (40), which implies questioning the use of CD63 as a standard EV marker (48). Finally, the expression of CD63 is susceptible to SCORT regulation leading to the blockage of the budding of this EV subset (49) and this mechanism could hypothetically be more frequent in urine-derived EVs. Regarding to plasma-derived EVs, the detection of the opposite pattern (CD133−/CD63+) in the EVs obtained with *standard* protocol unravel the isolation of a specific EV fraction, being probably only exosomes (46).

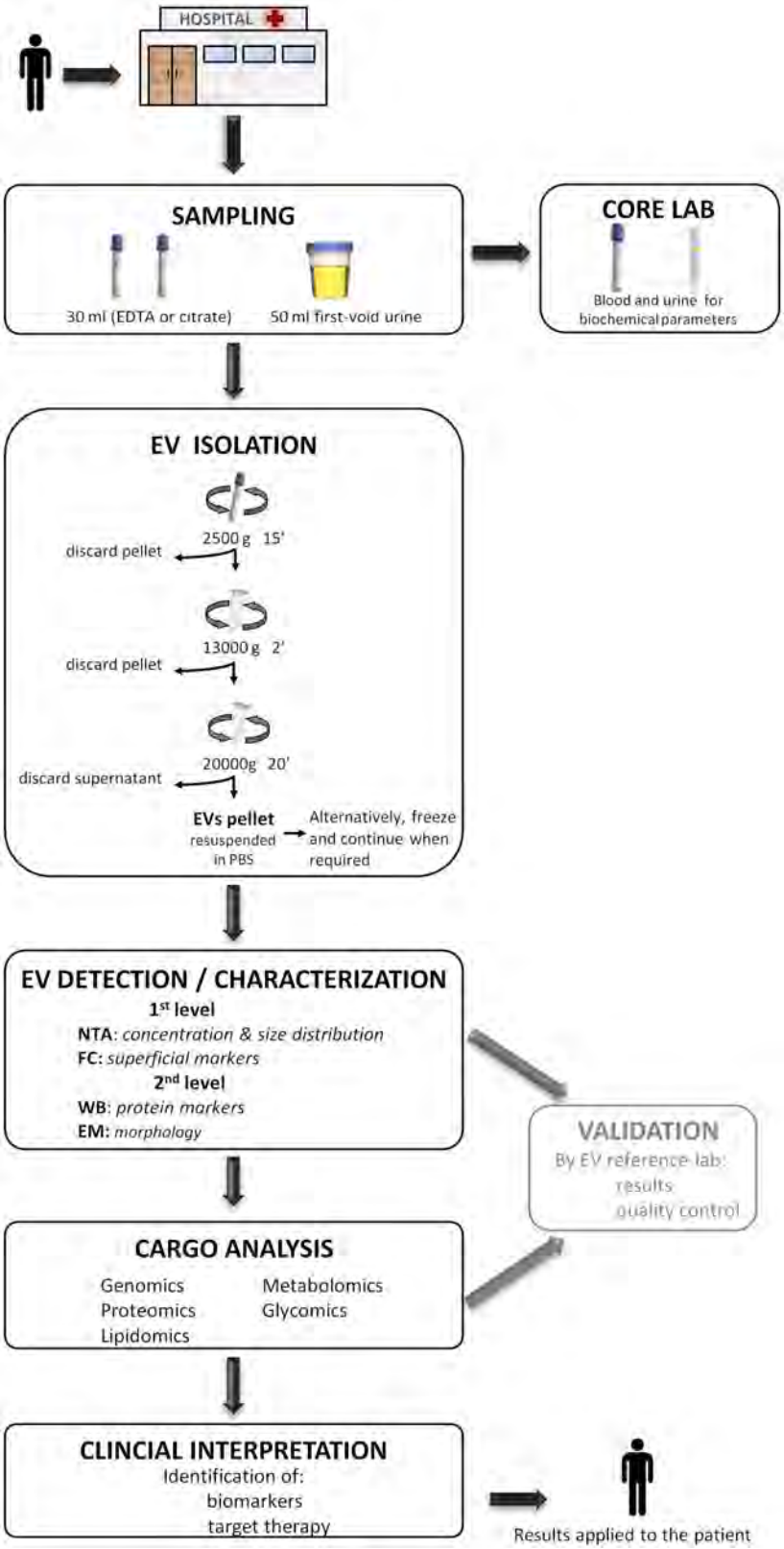

FIGURE 4 | A proposed workflow for the study of extracellular vesicles (EVs) in a hospital setting.

(*Continued*)

FIGURE 4 | Continued
Patients visited during the morning in the hospital, preferentially on fasting, undergo sample collection of 30 ml of blood (EDTA or citrate) and 50 ml of the first void urine. Immediately, 15 ml of blood and 40 ml of urine are destined to the EV isolation protocol to obtain a pellet and the rest 15 and 10 ml are sent to the core laboratory to analyze biochemical parameters. The obtained EV pellet resuspended in PBS could optionally be frozen at −80°C and continue when required. Next, the detection/characterization of EV is divided in two levels for quantification, size [nanoparticle tracking analysis (NTA)], and initial characterization with flow cytometry (FC) followed by an extensive description with western blot (WB) and electronic microscopy (EM). Subsequently, the analysis of EV cargo with several omics platforms allows the identification of specific compounds carried by EVs. EVs detection and their cargo analysis could optionally be referenced, at least during the initial setting of this workflow, to an expert EV laboratory in order to provide a validation of the results and pass a quality control test. Finally, the detected molecules are interpreted in the whole context of the patient with the aim of identifying biomarkers or a target for a putative therapy. The results provided by the study of EV are applied back to the patient improving the diagnosis or course of the disease.

Regarding the RNA concentrations yielded by the different EV isolation methods, we have observed great variability. Although Taylor and colleagues conclude that *exoquick* isolates more than, among other methods, ultracentrifugation (32), we have observed, unexpectedly, that the RNA concentrations obtained with the different methods are very similar, despite the notable differences in EV concentrations. Surprisingly, high RNA concentrations were obtained from urine (especially when using the *salting out* and *standard* methods), concentrations similar to or even higher than those obtained from plasma and serum, regardless of EV concentrations being between 10 and 20 times lower. These results lead us to think that, as we have not used RNAses, we are measuring the concentration not only of the RNA contained in the EVs but of the free RNA. In a position paper of the International Society of Extracellular Vesicles, the authors suggest that the use of RNAses only removes the free RNA not specifically bound to EVs, while their use in combination with proteases also removes the nucleoproteic complexes (50). In any case, if the final objective is to use the RNA as a source of potential biomarker, we believe that it would be useful to preserve not only the RNA inside the EVs but the RNA stuck to their membrane.

EVs FROM BENCH TO BEDSIDE

The importance of the study of EVs in a hospital setting to complement the diagnosis and prognosis of several diseases has been well demonstrated (51–53). Moreover, their application in therapeutic approaches has already been tested in clinical trials with promising results (54). Nonetheless, we believe that the workflows from the collection of the samples aimed at the isolation, processing, and characterization of EVs to yield significant results to be applied on patients need to be urgently standardized. Specifically, the different isolation method can yield different types of EVs and, thus, omics studies performed on them could give incomparable results. Besides, not all methods are applicable in a hospital setting.

With aim of contributing to this debate and in accordance to the results of the present work, we consider that the *centri13000* method is the most suitable one to be used in a hospital setting as (a) it requires a simple infrastructure (and does not require ultracentrifuge) that is available in any general laboratory, (b) isolates EVs with similar characteristics to the ones isolated with the *standard* method but in higher concentrations, (c) it recovers not only small EVs as standard does but also the largest, and (d) in analysis with FC and WB showed less contamination when comparing with *centri2500*. We concur with Deun and colleagues (55) in that it is necessary for a validation of the isolation procedure and we propose that this validation could be carried out in referent laboratories lead by group with great expertise in the study of EVs. The results obtained in the hospital setting should be compared to those obtained by the reference lab to assure a quality control. On the other, for the posterior detection and characterization of EVs, we recommend to analyze them with at least one quantification method (NTA or FC) and one characterization method (WB, EM, or FC) as they provide complementary information. **Figure 4** summarizes a proposed workflow based on the discussion above.

To conclude, the isolation of EVs, at least for plasma-derived ones, through differential centrifugation at medium speed (*centri13000*) and their posterior analysis with at least one quantification method (NTA, for example) and another characterization method (FC or WB, for example) could fit in a workflow that goes from the patient to lab and all the way back to the patient and would contribute to face several health problems.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Jounal/10.3389/fimmu.2015.00050/abstract

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REVIEW PAPER

Models for Studying Myelination, Demyelination and Remyelination

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Abstract One of the most widely studied demyelinating diseases is multiple sclerosis, which is characterised by the appearance of demyelinating plaques, followed by myelin regeneration. Nevertheless, with disease progression, remyelination tends to fail, increasing the characteristic neurodegeneration of the disease. It is essential to understand the mechanisms that operate in the processes of myelination, demyelination and remyelination to develop treatments that promote the production of new myelin, thereby protecting the central nervous system. A huge variety of models have been developed to help improve our understanding of these processes. Nevertheless, no single model allows us to study all the processes involved in remyelination and usually more than one is needed to provide a full picture of related mechanisms. In this review, we summarise the most commonly used models for studying myelination, demyelination and remyelination and we analyse them critically to outline the most suitable ways of using them.

Keywords Cell culture · Multiple sclerosis · Experimental models · Demyelination · Remyelination · Neurodegeneration

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Introduction

Myelin is a lipoprotein structure produced by two types of cells: oligodendrocytes (OLs) in the central nervous system (CNS) and Schwann cells in the peripheral nervous system. These cells are capable of generating myelin sheaths that coat axons; this, on the one hand, enables proper transmission of the nerve impulses, and on the other, provides an appropriate environment for the survival of axons, supplying the necessary nutrients and maintaining axonal homoeostasis (Bando et al. 2008). Myelin sheaths are crucial for the development and maintenance of our brain, and therefore, improving our understanding of the processes involved in their generation and repair are a clear goal in the neuroscience field.

In certain diseases, myelin is damaged, impairing the transmission of nerve impulses and compromising axon survival, producing clear clinical symptoms. Myelin-related disorders can be divided into two large groups, dysmyelinating and demyelinating diseases. The first group are related to an inappropriate production of myelin, while in the second, myelin is appropriately produced, but is damaged by the disease, in a process called demyelination. The most common disease in the second group is multiple sclerosis (MS).

When a demyelinating lesion occurs, myelin repair mechanisms are activated and the lesion is repaired with the production of new myelin. Oligodendrocyte progenitor cells (OPCs) are responsible for initiating this process. To do this, OPCs migrate towards lesions, proliferate and differentiate into OLs, which extend their membrane to recoat the axons (Ffrench-Constant and Raff 1986; Gensert and Goldman 1997; Rodriguez 2003; Watanabe et al. 2002).

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This process of remyelination enables, on the one hand, the restoration of the saltatory conduction of nerve impulses, and on the other, guarantees the maintenance of the necessary conditions for axon survival. After repeated cycles of demyelination/remyelination, however, the remyelination efficiency decreases and fails to restore axon function. Although the reasons for remyelination failure are not well understood, patient age, a hostile environment in lesions (e.g. cellular debris and inflammation), and progression of the disease itself may cause errors in the migration, proliferation and differentiation of OPCs (Franklin 2002), that result in a poor or ineffective remyelination. As a consequence, axons remain unprotected and they subsequently degenerate, promoting neurodegeneration, as occurs in advanced stages of MS (Ben-Hur 2011).

Increasing our knowledge of these processes is key to improving our understanding of the disease mechanisms and to developing therapies that increase remyelination capacity. Two main strategies are currently used to enhance remyelination: (1) the implantation of cells capable of generating myelin (exogenous therapy), aiming to repopulate the OPC-depleted tissue in the CNS, and (2) the stimulation of endogenous cells, to improve their differentiation and/or remyelination capacities (Miller and Fyffe-Maricich 2010; Zhang et al. 2011).

In order to deepen our understanding of myelin formation, we need to use experimental models of the demyelination and remyelination processes. Such models would be very useful to test the effect of potential therapies seeking to promote remyelination and also prevent axonal degeneration, which is the final goal in the field of demyelinating diseases. We should bear in mind that no model, no matter how sophisticated, can mimic all physiopathological processes that occur in humans. Nevertheless, there are models that simulate several aspects of myelin-related diseases and enable us to study myelination, demyelination and remyelination.

The aim of this review is to present, in order of complexity, the most commonly used models developed for studying the different pieces of the myelin puzzle, and describe their main characteristics and uses.

In Vitro Models

OPC Culture

During myelin production, OPCs differentiate to OLs, which are the cells responsible for creating myelin sheaths. The isolation and culture of OPCs can be a helpful tool for understanding the mechanisms involved in the development of myelin and for studying the effect of new therapies on these types of cells in relation to migration, differentiation, survival and proliferation (Chen et al. 2007; Dincman et al. 2012).

Many types of tissue have been used as a source of OPCs, including cryopreserved umbilical cord (Kurtzberg 2011), foetal brain (Monaco et al. 2012), brain biopsies and embryonic stem cells (Brüstle et al. 1999). These sources make it possible to work with human OPCs, but they have the drawback that samples are difficult to obtain and their use raises ethical concerns. Due to these factors, induced pluripotent stem cells (iPSCs) have emerged as an alternative source for obtaining human OPCs (Ogawa et al. 2011). The generation of iPSCs not only allows us to work with human-derived OPCs but also with large numbers of cells. Moreover, iPSCs have a potential therapeutic role since they can be obtained from patients themselves, allowing allogeneic transplantation. For research purposes, another option is to obtain OPCs from the optic nerve (Shi et al. 1998) or brain cortex of young and adult rodents (Wernig et al. 2008).

A variety of methods have been proposed for isolating OPCs: using an oligodendrocyte selection kit (Pesheva 2006), magnetic-activated cell sorting (Dincman et al. 2012), manual sorting (Medina-Rodríguez et al. 2013), immunopanning (Dugas and Emery 2013), fluorescence-activated cell sorting (Chen et al. 2007) and generating an immortalised cell line (Merrill 2009). All these methods can achieve good cell quality and high OPC-content cultures. Therefore, the method should be selected based on the equipment and resources of each lab. Once the OPCs have been isolated, differentiation, proliferation, survival or migration assays can be performed.

For instance, to characterise the ability of a drug to promote cell migration, we can measure the number of cells that cross the membrane after culture in transwells. In order to characterise each stage of OL differentiation after the administration of a treatment, various types of markers can be used for specific proteins, including platelet derived growth factor (PDGFR) α, neuron–glial antigen 2 (NG2) and A2B5 for OPCs, 2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNP), O1 and O4 for pre-OLs; myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG) for OLs; and oligodendrocyte lineage transcription factor 2 for all OL-lineage cells. For survival or proliferation, specific markers of cell death (caspase-3) or cell proliferation (5-bromodeoxyuridine) can be used respectively. Then, results can be analysed by fluorescence microscopy. Alternatively, flow cytometry can be used, facilitating multiparametric analysis, though it does not enable visualisation of cell morphology. Gene expression analysis can also be used to characterise the culture (Dugas et al. 2006; Robinson et al. 2014).

This in vitro model based on OPC culture is simple, low cost and high throughput. It represents a first step towards ascertaining whether treatments or the manipulation of a molecular pathway have positive effects on the cells responsible for myelination and remyelination, as in the work of Syed et al. (2013) who demonstrated that the inhibition of phosphodiesterase 4 promotes OPC differentiation. Moreover, since the cells are not influenced by other types of cell or the environment, exposure of these cells to a drug simplifies the interpretation and understanding of results (Merrill 2009). With this approach, we can lay the foundations for further work with more complex models.

"Axon-Based Models": Co-cultures

These models of myelination involve obtaining axons, either natural or artificial, on which to test the myelinating ability of cells under study, as a potential therapy in demyelinating diseases. It is possible to administer a drug as a treatment together with the cells, and then study its influence on myelin production by these cells. Below, we describe two methods for obtaining axons: from explants and by producing synthetic axons.

"Natural" Axons

This model is based on culturing CNS explants (mainly from rat or mouse embryos) in order to allow axons to extend outwards. These axons serve as a substrate for myelination by exogenous cells. Given differences between types of explants, the best source depends on the type of experiments to be conducted. One possibility is to obtain dorsal root ganglion explants (Päiväläinen et al. 2008). Nevertheless, this type of explants has limitations: firstly, dorsal root ganglia are not CNS neurons and, secondly, their axons extend only a short distance (Chen et al. 2010; Watkins et al. 2008). To improve our understanding the CNS myelination, spinal cord explants is one of the most widely used sources. A protocol improved by Thompson et al. (2006) made it possible to obtain spinal cord in which cell somas remained in the explant and axons extended rapidly from the explant, this facilitating the observation and measurement of myelination. Depending on the method used to culture the spinal cord explant, axons can also be obtained from motoneurons (Hedvika et al. 2012; Hyung et al. 2015) or sensory neurons (Callizot et al. 2011). Another approach is the use of whole brain spheroid aggregates. These are 3D explants that maintain the complexity of in vivo biology and represent a halfway house between in vitro and ex vivo models. They have multilayered myelin and can be used for de- and remyelination studies (Vereyken et al. 2009).

Once the explants have been cultured, myelinating cells are added. Using these models, it is possible to assess the capacity of myelinating cells to form myelin around axons, as well as the effect of drugs on these cells in the presence of neuron–glia interaction. It is also possible to add to our understanding of the mechanisms involved in the myelination process, as illustrated by the work of Chan et al. (2004) that demonstrated the involvement of nerve growth factor in the control of myelination. The effect of a cell therapy can be analysed by measuring the quantity of myelin produced by cells with immunofluorescence microscopy of the axon (neurofilaments) or the myelin (myelin basic protein, myelin oligodendrocyte glycoprotein for more mature myelin and CASPR that can suggest more compact myelin). To facilitate the monitoring of myelination, it is possible to add green fluorescent protein-labelled OPCs (Chen et al. 2010). In parallel, electron microscopy can be performed to determine the myelin g-ratio, which is the ratio between the inner and the outer diameter of the myelin sheath, and is used as an indicator of optimal myelination (Chan et al. 2004; Chomiak and Hu 2009).

This is a myelination model that allows us to study interactions between neurons and myelinating cells during the myelination process. We need to bear in mind, however, that in these explants, besides neurons, there are other types of cells that can also influence the myelination process, producing uncontrolled effects (Pang et al. 2012). For this reason, some authors classify these as ex vivo models.

Synthetic Axons

In order to avoid the uncontrolled interactions between myelinating cells and other cells types present in the explants, variants of the previous model have been developed that consists in the use of inert or synthetic axons (Lee et al. 2012). In order to obtain inert axons, after purification from a biopsy, the axons must be fixed with paraformaldehyde (Rosenberg et al. 2008). Alternatively, synthetic axons can be obtained by producing artificial nanofibres. For this purpose, a wide range of materials have been tested, from glass microfibres coated with a glial cell matrix (Bullock and Rome 1990), passing through vicryl microfibres, coated with extracellular matrices containing molecules to promote cell attachment (Howe 2006), to polystyrene (Lee et al. 2013; Seonok Lee et al. 2012) or polycaprolactone (Diao et al. 2015) nanofibres designed using electrospinning. A modification of this synthetic axon model uses micropillar arrays, formed in a 96-well plate, which consist of 50-µm-diameter conical structures that cells are able to myelinate (Mei et al. 2014).

Artificial fibres can be coated with selected substances (from proteins to microRNAs) (Diao et al. 2015), in order to study in depth their effect on myelination (Lee et al.

2013). This facilitates the subsequent design of remyelinating therapies based on these substances.

Ex Vivo Models

Organotypic Cultures

These types of cultures involve the growing of tissue in three dimensions, mimicking the structure and cell types of living organs, making them an intermediate step between cell cultures and animal models. These cultures enable the development of models for certain neurological diseases such as ischaemia, Parkinson's disease and Huntington's disease, as well as MS (Daviaud et al. 2013).

Several CNS structures including the brain, cerebellum and spinal cord can serve as the source for organotypic cultures (Kipp et al. 2012), each with their own characteristics. The cerebellum provides homogeneity in the type of axons present, mostly Purkinje cell axons, while the brain and the spinal cord have more axonal variety in terms of type and diameter. On the other hand, the spinal cord may be a good choice for OPC migration studies (Zhang et al. 2011).

Briefly, with the help of a vibratome or tissue chopper (McIlwain), approximately 300-μm slices of tissue are obtained and then cultured on membranes (designed for this type of culture) for subsequent demyelination. The first report of induced demyelination dates from 1959, in which Bornstein and Appel (1959) described successful demyelination of a cerebellar organotypic culture by adding serum from animals with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Nowadays lysophosphatidylcholine, a detergent that mainly destroys myelin, is used as a demyelinating agent (Birgbauer et al. 2004). After removing the detergent, the OPCs in the culture proliferate in response to the demyelination and differentiate to OLs that are capable of regenerating the myelin, enabling remyelination studies (Zhang et al. 2011).

This type of model allows us to assess the regenerative capacity of endogenous cells and speed of regeneration achieved under the influence of different drugs. It is also possible to analyse remyelination by cell therapy. For this, cytosine arabinose should be added to the culture to suppress the proliferation of endogenous cells (Nishimura et al. 1985), in order to suppress the remyelinating potential of the OPCs present in the tissue.

Several techniques can be used to measure remyelination. Confocal microscopy is useful to semi-quantitatively assess the area occupied by myelin-coated axons (compared to the total area of axons) (Pang et al. 2012). For this, at the end of the culture period, the tissue is fixed and immunofluorescence staining is performed to label axons and myelin. The major inconvenience of this method is that it is highly dependent on the analysis of images. Attempting to address this problem, Zhang and collaborators described an automatic quantification method using Image-Pro Plus software (Zhang et al. 2011). In addition, culture growth can be followed with time-lapse imaging (Schnädelbach et al. 2001; Zhang et al. 2011). As an indicator of remyelination quality, we can measure the distance between nodes of Ranvier, the so-called internodal length: the shorter this distance, the poorer quality the remyelination. Finally, g-ratios can also be calculated as a measure of remyelination quality. Both of these indicators can be calculated with data from electron microscopy images.

To achieve a favourable culture growth, young animals should be used. Nevertheless, it should be also taken into account that demyelinating diseases do not tend to occur in early stages of development, and this may have an impact on the conclusions obtained. Finally, we have to take into account that though the structure of the original tissue is maintained, these models do not replace in vivo models, rather they are useful for screening treatments in advance of in vivo testing, thereby reducing the number of animals used subsequently (Daviaud et al. 2013). In line with this, Meffre et al. (2015) demonstrated the importance of these kinds of models in drug discovery, having used them for the development of therapies targeting liver X receptor alpha.

In Vivo Models

Zebrafish

The zebrafish is a vertebrate animal that has been widely used in developmental studies given that it has a short life-cycle and is transparent, making it possible to visualise internal structures noninvasively. Moreover, it has been described that myelination in this fish species is similar to that in mammals (Buckley et al. 2008). In fact, it has homologous genes involved in the myelination process (Dubois-Dalcq et al. 2008). For these reasons, embryos of this species have been used in myelination studies (Kim et al. 2011) and its larvae have been used in drug screening.

Further, transgenic zebrafish have been developed in which OLs (Kirby et al. 2006), or both OLs and Schwann cells (Jung et al. 2010), express membrane-targeted green fluorescent protein, making these cells visible. Such models are useful for studying myelination in vivo (Jung et al. 2010), but do not allow analysis of remyelination due to the lack of physiological demyelination. To perform remyelination studies in this type of model, demyelination has to

be achieved, for example, by damaging myelin with laser microsurgery (Kirby et al. 2006), or by producing transgenic individuals, such as the system reported in 2013 by Park's research group, in which OLs die after exposure to metronidazole (Chung et al. 2013). In these models, the remyelination process starts after the microsurgery or stopping the drug treatment, respectively.

Both myelination and remyelination processes can be monitored in vivo by time-lapse confocal microscopy without the need to kill the animal, provided that OLs express a fluorescent protein (Kirby et al. 2006). In addition, post-mortem immunofluorescence can be performed to identify the proteins of interest.

This model provides a rapid method for assessing candidate agents for the treatment of demyelinating diseases, given that it is possible to monitor OLs in vivo (Buckley et al. 2010; Dubois-Dalcq et al. 2008). This was illustrated by Franklin's research group in 2010, with the development of a screening platform based on this type of model for identifying the most promising compounds to be tested later in mammalian models (Buckley et al. 2010). These models are not suitable, however, for testing cell therapy, given the technical difficulties involved.

Mammalian Animal Models

A great variety of models allow us to study remyelination in mammals. The main advantage of using mammal models is that some biomolecular pathways are shared with human forms of disease. In this review, we will focus only on murine models, which are the most widely used for studying myelin-related processes. Such models have been developed for studying pathological processes associated with demyelinating diseases and remyelination, and can be classified into three types: models of toxicity, models of viral infection and models based on immune response. Given that there are several high-quality reviews of mammalian models (Baker and Amor 2015; Kipp et al. 2012; Star 2012), we will only list them and briefly outline some of their characteristics.

The ways of obtaining data are similar for all the murine models listed, with some exceptions that will be specified later and can be summarised as follows:

- During the development of the model, magnetic resonance imaging and two-photon imaging can be used for monitoring animals (Rassul et al. 2016).
- After killing animals, histopathological studies can be performed to detect myelin or different types of CNS cells in tissue with, for example, Luxol Fast Blue or immunohistochemical and immunofluorescence analyses. The remyelination quality can be assessed, by determining the g-ratio of axons by electron microscopy. Another possibility is to extract CNS tissue, make an emulsion from this tissue and assess the cell content in the emulsion by flow cytometry or gene expression analysis (Robinson et al. 2014).

Models of Toxicity

Toxin-Induced Local Lesions These are models of localised acute cytotoxicity, in which OLs are depleted (Magalon et al. 2007) following an injection, by stereotaxy, of ethidium bromide (EB) or lysophosphatidylcholine (LPC), among other agents. EB is an intercalating dye that induces the loss of OLs and astrocytes, but does not affect axons (Kuypers et al. 2013). LPC alters membrane composition, specifically of OLs, due to fusogenic properties of their membrane; this triggers damage to the myelin (Allt et al. 1988; Vereyken et al. 2009). As a consequence of the demyelinating lesion, microglia and macrophages move towards the lesions generated, there is reactive astrogliosis, axonal homoeostasis is disturbed, and OPCs proliferate and migrate to the damaged tissue (Keough et al. 2015). There is no consensus on whether or not this model shows concomitant demyelination and excessive inflammation (Kuypers et al. 2013; Miron et al. 2011). Further, lesions can be directed to different regions of the CNS and peripheral nervous system, including the dentate gyrus (Babri et al. 2015), sciatic nerve (Bondan et al. 2009), spinal cord (Keough et al. 2015) and centrum semiovale (Dousset et al. 1995). Such models have been used widely in primates, as well as in rodents.

These toxin-induced models are highly reproducible (Miron et al. 2011) and useful for studying the effect of different drugs on remyelination alone, in the absence of inflammation; this method was applied by Goudarzvand and collaborators, for studying the involvement of vitamins E and D3 in the enhancement of remyelination (Goudarzvand et al. 2010). It needs to be taken into account, however, that there is necrotic damage around the injection site (Dousset et al. 1995; Miron et al. 2011).

Models of General Toxicity These models are based on the production of demyelinating lesions by neurotoxic, chemical, or biological agents administered through the diet. The most widely used toxin is cuprizone, a copper-chelating agent that induces OLs apoptosis, and as a consequence, demyelination. Though the mechanism involved remains unclear, it has been proposed that cuprizone produces errors in the mitochondrial respiratory chain (Kipp et al. 2009). In contrast to EAE, that will be explained further below, T cells do not play any role in the generation of this model, and not all the regions of the brain are affected in the same way (Torkildsen et al. 2008). By using different lengths of cuprizone treatment, it is possible to produce an

acute or a chronic model (6 or 12 weeks of treatment, respectively). In both models, after stopping the cuprizone treatment, there is spontaneous remyelination of the lesion, this being slower and more limited in the chronic model (Acs and Kalman 2012; Kipp et al. 2009). A variation of these models has been developed, using cuprizone in combination with rapamycin, achieving a complete demyelination and slower remyelination than with cuprizone alone (Sachs et al. 2014).

This type of model is simple and reproducible, but we must bear in mind the age, sex and the species of animals used, since this can have an impact on the results (Kipp et al. 2009). For instance, remyelination has been found to be slower and more limited in old animals (Torkildsen et al. 2008). These models make it possible to study demyelination and remyelination processes that occur in the CNS and the effect of drug and cell therapies (Acs and Kalman 2012). Note that, to study cell therapies, it is necessary to administer cells after withdrawing cuprizone from the diet in order to avoid damaging the injected cells. Further, we should emphasise that precursor cells are not affected by cuprizone and hence they will compete with the administered cells to repair the lesions (Kipp et al. 2009). This model has helped to demonstrate the protective role of rolipram in cuprizone-induced demyelinated animals (Sun et al. 2012).

Models Based on Viral Infection

These models are based on the hypothesis that some viral infections may cause demyelinating diseases such as MS. The most commonly used viruses are the Semliki Forest virus (Smithburn et al. 1946) and Theiler's murine encephalomyelitis virus (Theiler 1934). Semliki Forest virus was first isolated in mosquitoes and rarely affects humans. The virus can infect OLs and, due to its highly virulent nature, the M9 mutant also usually causes the death of the host. For this reason, the avirulent A7 strain is more frequently used, showing its effect through the immune response against infected cells and not through virus replication with subsequent cell death. Theiler's murine encephalomyelitis virus leads to neuropathological processes (such as paralysis and encephalomyelitis) in a wide range of animal species. The most commonly used strains include the virulent GDVII and the less virulent Daniels and BeAn strains.

Models of viral infection allow us to explore the potential effect of immunomodulatory and remyelination therapies on CNS cells infected by a neurotropic virus (Baker and Amor 2015). This feature is interesting since it has been suggested that MS may be caused by a virus (Oskari Virtanen and Jacobson 2012). On the other hand, these models are not appropriate for testing cell therapies, as the virus would have a tropism for injected cells, being affected in the same way as endogenous cells. To date, they have not been extensively used and this is attributable to a range of factors: the long incubation periods between infection and the onset of symptoms, the high mortality rates among infected animals, in particular when we use virulent strains, and the technical difficulties associated with the manipulation of viruses. Nevertheless, their application has contributed, for example, to the discovery that a 40-nucleotide single-stranded DNA aptamer has the ability to promote remyelination of CNS in mice after Theiler's virus infection (Nastasijevic et al. 2012).

Models Based on Immune Response: Experimental Autoimmune Encephalomyelitis

The first reference to this experimental model dates from 1933, when Berry's research group, after several consecutive intramuscular injections of brain emulsion, succeeded in causing inflammation and demyelinating lesions in the CNS of monkeys (Rivers et al. 1933). At that time, the mechanism involved had not been identified, but now it is known that this condition is mediated by specific T cells against myelin antigens, and that it has clinical and histopathological similarities to MS (Zamvil et al. 1986). In fact, this is the experimental model that exhibits most similarities to the human disease and the most commonly used for studying remyelinating drugs in this disease.

The features it shares with MS include the destruction of myelin, the development of lesions over time and across the CNS, though mainly perivascular in scattered foci, and the presence of immunoglobulins in both the cerebrospinal fluid and the CNS. On the other hand, the processes of demyelination and remyelination in this type of model are less extensive and more acute than in humans (Miller and Fyffe-Maricich 2010).

There are two subtypes of this model. The first is the active one, in which animals are immunised against a myelin peptide, together with Freund's adjuvant and pertussis toxin. Depending on the peptide, animal host and viral strain, numerous different models have been developed, and these are widely reviewed in the literature (Baker and Amor 2015; Kipp et al. 2012; Star 2012). The second subtype, the passive or the adoptive cell transfer model, is produced by injecting specific active lymphocytes against myelin, which are obtained from the lymph nodes of animals that have undergone active immunisation.

This is considered the gold standard model for the study of MS. It is used for preclinical proof of concept for new pathways and mechanisms of action in the pathogenesis of autoimmune diseases. Many of the findings with this model have been applied to treatments in patients. In fact, Susumu Tonegawa and Peter Doherty were awarded a Nobel Prize

for their work related to this model (Hohlfeld 2009; Steinman 2003). Nevertheless, we must be cautious since therapies proven to be effective in the animal model may not produce the same positive effects in humans (Constantinescu et al. 2011). Moreover, this type of model fails to predict adverse effects of treatments, especially in cases when treatments need to be long term, such as those for MS (Daviaud et al. 2013; Steinman and Zamvil 2005). Moreover, it is an expensive animal model, in terms of time and money, given the numbers of animals that are needed (due to variability) and its low throughput (Zhang et al. 2011). Its limitations are mainly related to a lack of understanding of all the mechanisms involved in the pathogenesis of the model, and the short time frame of the model for studying chronic conditions. Finally, this is the most complex model of all those mentioned in this review and its complexity sometimes makes the interpretation of findings difficult (Moreno et al. 2012).

As this review is focused on models for studying myelination–remyelination, we want to emphasise the most suitable type of model for this kind of research. Remyelination failure is characteristic of the chronic forms of MS. For this reason, models of chronic EAE are particularly suitable for studying remyelination due to the great similarities with the secondary progressive MS (Constantinescu et al. 2011). These are produced by myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide in Dark Agouti rats and C57BL/6 mice (Lorentzen et al. 1995; Mendel et al. 1995).

The main information obtained from this type of model is the animals' clinical score. This information is subject to observer bias; therefore, it is essential to carry out double-blind experiments as well as robust statistical analyses (Fleming et al. 2005). Furthermore, the findings obtained with this model should be complemented with experiments carried out using some of the other aforementioned models.

Other Models

In 2015, Gocke's research group published a study that combined an EAE model with a toxicity model, with the goal of obtaining a model that overcomes the problems of using these models on their own. In this new model, demyelination occurs in the absence of neurodegeneration which, according to the authors, makes it possible to assess remyelinating therapies that have previously been difficult to study given the neurodegeneration in EAE (Baxi et al. 2015).

Lastly, there are models that are based on the use of genetically modified animals, in which T cells express specific receptors of myelinating cells, in order to produce demyelinating lesions. However, these models are not yet commercially available, are rarely used, and require long periods of development until the animals manifest clinical signs.

Selecting the Appropriate Model In this review, we have outlined models for studying the processes of myelination, demyelination and/or remyelination. There is a wide range of models and we believe that it is important to underline certain characteristics of the models considered, to help us to select the best models for our goals.

- *Myelination, demyelination and remyelination* Although the final goal is to promote the production of new myelin after the damage caused by some type of disorders, not all the models assessed allow us to analyse this directly. For example, the analysis of OPC differentiation after a treatment can give us important information about the process of differentiation in particular, such as, whether a drug works in the expected way, but does not help us determine whether the compound improves axon survival. We should remember that all the models provide complementary information needed to improve our understanding of the mechanisms related to remyelination processes and to build a picture of the whole process.
- *What question do we want to answer?* As has been emphasised, not all models can provide answers to all questions. For this reason, it is important to clearly define what types of data we require and seek to obtain them with the most suitable model. For example, if we want to analyse the molecules involved in the wrapping of axons after a certain treatment, an animal model would be too complex for this goal, and an axon co-culture would be more suitable. Table 1 summarises the main characteristics of all the models reviewed.
- *Better to use more than one model* It is advisable to use more than one model, since different models can provide complementary information that increases the quality of the results. Each model has strengths and weaknesses, and provides different types of data, which well used, can be put together in order to obtain more robust conclusions to guide the development of new therapies.
- *The most complex is not always the best* The greater the complexity of the model, the closer to the human disease. Nevertheless, understanding the mechanisms underlying remyelination with simpler models may help us identify and improve our understanding of certain processes that could lead to the development of remyelinating therapies.
- *From simple to complex models* The simplest models allow us to test more molecules/therapies, since it is easier to obtain larger sample sizes with fewer ethical constraints. Moreover, such models are easier to

Table 1 Summary of the main characteristics of the model types reviewed

	In vitro models			Ex vivo model	In vivo models				
	OPCs	Spinal cord explants (OPCs)	Synthetic axons	Organotypic cultures	Zebrafish	Local toxicity	General toxicity	Viral infection	EAE
EAE: experimental autoimmune encephalomyelitis	+++	++	++	+ +	+++	+	+	+	+
Short periods of model development	+++	+++	+++	+++	++	++	+	+	+
Presence of inflammation	0	0	0	0	+	+	+	+++	+++
Testing of drugs	+++	++	++	+++	+++	+++	+++	+++	+++
Testing of cell therapies	0	++	+++	++	+	++	+	+	+++
Ethical constraints	++	++	++	++	+++	+++	+++	+++	+++
Demyelination studies	0	0	0	++	++	+	+	+	+++
Remyelination studies	0	0	0	+++	++	++	+++	+++	+++
Myelination studies	+++	+++	+++	+	+++	0	0	0	0
Level of complexity	+	++	++	++	+++	+++	+++	+++	+++
Closeness to humans (inflammation, lesion type, structure of processes)	0	0	0	0	+	++	++	+++	+++

"+++" indicates the highest value for that characteristic and "+" the lowest. "0'' means that this characteristic is not present

EAE: experimental autoimmune encephalomyelitis

manage and less expensive. The compounds that have yielded positive results can then be analysed in more complex models, which better mimic the mechanisms occurring in humans. In this way, as we move to more complex models, we reduce the number of candidate agents that are eventually going to be tested in clinical trials (Fig. 1).

- *We cure mice; what about humans?* Treatments studied in experimental models with positive results do not always show the same effects in human patients, being found to be ineffective or to produce serious side effects. It is necessary to remember that we are working with in vitro, ex vivo and in vivo experimental models and extreme care must be taken in extrapolating the

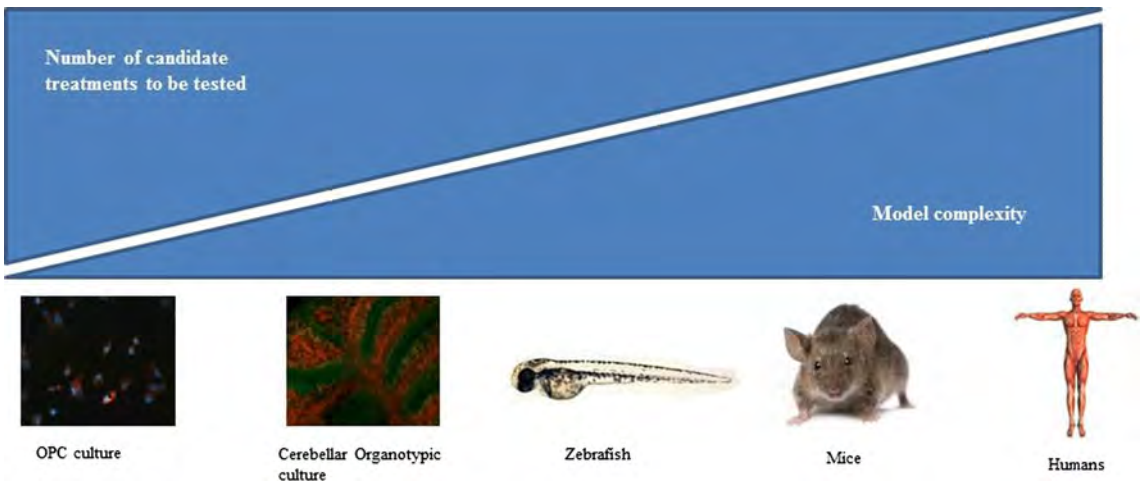

Fig. 1 Schematic representation of the complexity and number of candidate treatments that can be tested in each model. From *left to right* in vitro models, represented by an image of OPC culture stained with DAPI (*blue*) and MBP (*red*); ex vivo models, represented by an image of cerebellar organotypic culture stained with NFL (*green*) and MBP (*red*); increasingly complex in vivo models, represented by images of a Zebrafish and a mouse; and at the end of the process, humans (Color figure online)

results to humans, since no model is able to exactly reproduce processes that take place in human diseases.

Concluding Remarks

In this review, we have outlined some of the most commonly used in vitro, ex vivo and in vivo models for the study of myelination, demyelination and remyelination. Once the aim of a research study has been defined, it is essential to select the best model for building a picture of the processes that we seek to study.

Clearly, simple models are the easiest experimentally, less expensive and less time-consuming. Nevertheless, it is impossible to draw far-reaching conclusions from a simple model, such as OPC culture, in which demyelination and remyelination cannot be studied. To address this, a model in which more elements are involved, such as the EAE animal model, should be used. On the other hand, its complexity makes it difficult to obtain definitive conclusions due to the limited understanding of the pathways involved.

In the future, it would be helpful to have models that were relatively simple to handle, but at the same time, able to reflect the complexity of the process, including several types of cells, inflammation, 3D structures, etc. Unfortunately, such models have not yet been developed, but the organotypic slice culture is a candidate, given that it is an ex vivo model, that maintains the 3D structure of the tissue from which it was derived, grows under controlled conditions, and can be used to test many drug and cell therapies. Nevertheless, we should not finish this review without underlining that the use of more than one complementary model is recommendable, in order to extract specific conclusions from each one and provide the most realistic picture of the processes of myelination, demyelination and remyelination.

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Compliance with Ethical Standards

Conflict of interest Osorio-Querejeta, I., Sáenz Cuesta, M., Muñoz-Culla, M. and Otaegui, D. declare that they have no conflict of interest.

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frontiers in Molecular Neuroscience

MINI REVIEW
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Therapeutic Potential of Extracellular Vesicles for Demyelinating Diseases; Challenges and Opportunities

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Multiple Sclerosis is a demyelinating disease of the central nervous system for which no remyelination therapy is available and alternative strategies are being tested. Extracellular vesicles (EVs) have emerged as players in physiological and pathological processes and are being proposed as therapeutic targets and mediators. More concretely, EVs have shown to be involved in myelination related processes such as axon-oligodendrocyte communication or oligodendrocyte precursor cell migration. In addition, EVs have been shown to carry genetic material and small compounds, and to be able to cross the Blood Brain Barrier. This scenario led scientists to test the ability of EVs as myelin regeneration promoters in demyelinating diseases. In this review we will address the use of EVs as remyelination promoters and the challenges and opportunities of this therapy will be discussed.

Keywords: remyelination, exosomes, myelin, multiple sclerosis, microRNAs, EAE, oligodendrocyte

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INTRODUCTION

Myelin is a membranous sheath produced by oligodendrocytes (Ols) in the central nervous system (CNS) that surrounds axons allowing the saltatory nerve impulse transmission. Moreover, myelin protects axons and contributes to the maintenance of its homeostasis. Myelin can be damaged in a physiological context, due to the normal aging process, but it can also be caused by pathological mechanisms. The latter scenario occurs in a wide variety of pathological situations, such as traumatic demyelination, leukodystrophies or multiple sclerosis (MS), being the last one the most common demyelinating disease. Although no specific auto-antigen has been identified yet, MS is considered a chronic autoimmune CNS disease that includes the breakdown of the Blood-Brain Barrier, inflammation, demyelination, oligodendrocyte loss, gliosis and axonal degeneration (Baecher-Allan et al., 2018). It is accepted that the activation of peripheral autoreactive effector $CD4^+$ T cells that migrate into the CNS attacking the myelin sheath is the main cause of MS appearance. Once in the CNS a second reactivation occurs in which other cells types such as B and $CD8^+$ T cells of the adaptive immune response, together with natural killers and microglia cells of the innate immune system contribute to the disease causing oligodendrocyte destruction, myelin loss, and an imbalance of the homeostasis of axons (reviewed in Baecher-Allan et al., 2018). This imbalance causes axon damage and an inefficient nerve impulse transmission. In the first stages of the disease, myelin can be restored, recovering normal electrical signal transmission. This is a complex process named remyelination in which a dynamic combination of different signaling

pathways and molecules such as growth factors, cytokines and chemokines are tightly regulated (Kuhlmann et al., 2008). To achieve remyelination, oligodendrocyte precursor cells (OPCs) need to (1) proliferate, (2) migrate into the lesions, and (3) differentiate to myelinating Ols that will generate new myelin (Miron et al., 2011). Nevertheless, with the progression of the disease this process tends to fail. It is not completely understood why remyelination capacity decreases with time, but a lack of OPCs, a poor migration of these cells or their impossibility to differentiate to Ols have been proposed (Franklin, 2002). In addition, it is increasingly recognized that age is not only a risk factor for neurodegeneration but also adversely influences regenerative processes and remyelination (Hampton et al., 2012). Moreover, some factors such as genetic background and diet are also involved in the reduction of the remyelination capacity (revised in Adamo, 2014).

To avoid neurodegeneration and promote neuroprotection, as well as the restoration of the fast saltatory conduction, the generation of new myelin is of extreme importance. The promotion of remyelination might protect axons avoiding their degeneration and probably improving patients' prognosis. Therefore, pharmaceutical companies and researchers that work in the field are trying their best to develop new remyelination therapies.

To this end, the replacement of the endogenous OPC population and the stimulation of endogenous OPCs to regenerate myelin are being analyzed, being differentiation a key point in this process (Hartley et al., 2014). Finally, targeting the immune system has been also pointed out as a therapeutic strategy to induce remyelination (Dombrowski et al., 2017; El Behi et al., 2017).

An ideal therapy should be able to cross the BBB and reach the CNS, target OPCs and not other cell types and should have minimal side effects. Owing to their natural capacity to affect cell proliferation and differentiation, and their potential to cross BBB, extracellular vesicles (EVs) have emerged as highly promising candidates for the treatment of demyelinating diseases, as discussed in detail in the following sections.

WHY EXTRACELLULAR VESICLES?

Intercellular communication is a key factor for the functioning and regulation of all biological processes. Apart from the two classical mechanisms – direct cell-to-cell communication and transfer of secreted soluble molecules –, in the last years extracellular vesicles (EVs) have been found to play a central role in intercellular communication.

Extracellular vesicles are membrane-bound particles secreted by cells. There are different types of EVs and the most common classification is based on their size and biogenesis (Raposo and Stoorvogel, 2013). EVs formed inside multivesicular bodies and released upon fusion of these bodies with the plasma membrane are called exosomes. Their main characteristic is to have a uniform size of between 30 and 150 nm, thus being the smallest EVs. On the other hand, those known as microvesicles, come from the evagination and direct budding from the plasma membrane. Microvesicles vary greatly in size, ranging generally from 0.3 to 1 μm in diameter; however, it must be noted that in many scenarios it can be difficult to separate exosomes from microvesicles purely based on their size (Willms et al., 2018). Another type of membrane vesicles are apoptotic bodies, which are 1–5 μm in size and were described many years ago and have different features to those derived from living cells (György et al., 2011). Currently, the generic term EV is used to refer to the complete set of secreted vesicles (Gould and Raposo, 2013). EVs play an essential role in indirect intercellular communication as their membrane, cytosolic proteins, lipids, metabolites and genetic material can be transferred between cells (Théry et al., 2001; Valadi et al., 2007). They can follow two different ways of integration: by direct fusion with the plasma membrane or by endocytosis (Morelli et al., 2004; Montecalvo et al., 2012).

Most cell types release EVs being secreted both in physiologic and pathogenic conditions. They can be isolated from many body fluids, including plasma and cerebrospinal fluid (CSF). EVs are involved in many biological processes, their capacity to regulate immune response and cell differentiation being the two most important processes in the context of this review (Robbins and Morelli, 2014). Moreover, EVs take part in the transmission of information across the CNS (Frühbeis et al., 2013b) and have been found to play a role in the regulation of synaptic activity (Fauré et al., 2006) and myelin sheath biogenesis (Marzesco et al., 2005; Bakhti et al., 2011), as well as in the repair of damaged neurons (Court et al., 2011).

The pathogenesis of several diseases has been shown to be linked to EVs, including cancer (Robbins and Morelli, 2014), neurodegenerative diseases (Basso and Bonetto, 2016; Thompson et al., 2016) and, of particular interest to this work, MS (Verderio et al., 2012; Sáenz-Cuesta et al., 2014a,b; Selmaj et al., 2017). The implication of EVs and their ability to carry messages from one cell to another suggests that the use of EVs as a drug delivery system or as a treatment, might be an interesting way of targeting and modulating the course of the disease. Moreover, the fact that EVs are able to cross the BBB makes them strong candidates for CNS disease therapy (Jan et al., 2017).

THERAPEUTIC POTENTIAL OF EVS FOR DEMYELINATING DISEASES

Several works have been published demonstrating the therapeutic potential of EVs. These works will be discussed in the following paragraphs and have been summarized in **Table 1**.

In some demyelinating pathologies, such as MS, the immune system is responsible for the damage caused to myelin. In fact, all the available treatments for MS are immunomodulatory or immunoregulatory drugs that prevent autoimmune attacks on the myelin sheath. In this way, the ability of exosomes isolated from pregnant mice serum or human periodontal ligament stem cells-derived exosomes to reduce the clinical score of the Experimental Autoimmune Encephalomyelitis (EAE), an animal model of MS, has been addressed by inhibiting the immune response, and more concretely by dampening Th1 response (Williams et al., 2013; Rajan et al., 2016). In addition,

TABLE 1 | Summary of therapeutic potential of EVs for demyelinating diseases.

Reference	EVs type	EVs Source	Isolation method	Principal experiment	Route of administration	Result
Williams et al., 2013	Exosomes	Virgin and pregnant mice serum	Ultracentrifugation	EAE	Intravenous	Stablished EAE supression.
Rajan et al., 2016	Exosomes	HPLSC culture supernatant	ExoQuick TC	EAE	Intravenous	Inmmunomodulation of EAE.
Zhuang et al., 2011	Exosomes	Glioblastoma culture supernatant	Sequential centrifugation steps	EAE	Intranasal	EAE inhibition.
Frühbeis et al., 2013a	Exosomes	Oli-Neu cultures supernatant	Sequential centrifugation steps	Oligodendrocyte-neuron co-culture	N/A	Exosomes mediated communication.
Krämer-Albers et al., 2007	Exosomes	Primary oligodendrocytes culture supernatant	Ultracentrifugation	Oligodendrocyte culture	N/A	Exosomes contain PLP, MBP, MOG and CNP.
Bakhti et al., 2011	Exosomes	Primary oligodendrocytes culture supernatant	Sequential centrifugation steps	Oligodendrocyte culture	N/A	Oligodendrocytes derived exosomes inhibit OPC differentiation.
Kurachi et al., 2016	Extracellular vesicles	MVECs culture supernatant	ExoQuick TC	Oligodendrocyte Precursor cell culture	N/A	OPCs survival, proliferation and motility.
Otero-Ortega et al., 2017	Exosomes	MSC culture supernatant	miRCURY Exosomes Isolation Kit	Subcortical ischemic stroke	Intravenous	Promotion of olifodendrocyte formation and remyelination.
Pusic and Kraig, 2014	Exosomes	Youth and Environmental Enriched rat serum	ExoQuick TC	Old rats	Intranasal	Enhanced myelin content.
Pusic et al., 2016	Exosomes	Environmental Enriched rat serum	ExoQuick TC	Demyeliantion hipocampal slice culture	N/A	Myelination increased and oxidative stress reduced.
Doeppner et al., 2015	Extracellular vesicles	MSC culture supernatant	PEG precipitation method	Ischemic stroke	Intravenous	Neuroprotection and neuroregeneration.
Drommelschmidt et al., 2017	Extracellular vesicles	MSC culture supernatant	PEG precipitation method	Perinatal brain induced inflammation	Intraperitoneal	Immunomodulation and reduction of micro- and astrogliosis.

the intranasal administration of curcumin-loaded glioblastoma-derived exosomes to EAE animals ameliorated the clinical symptoms of the model. Although the mechanism of action is not clear, the induction of immune tolerance and the apoptosis of activated immune cells are postulated to be behind this process. This data demonstrate that exosomes could work as anti-inflammatory drug delivery vehicles (Zhuang et al., 2011).

As was mentioned in the introduction, Ols are responsible for generating myelin that enwraps axons. The communication between Ols and axons is essential for the survival and functional maintenance of both. Interestingly, this communication between Ols and axons has been shown to be mediated by exosomes and, in addition, the interactions between Ols and axons might affect the cargo of exosomes (Frühbeis et al., 2013a). Moreover, when the cargo of exosomes released by Ols was analyzed, researchers found that those vesicles contained high levels of myelin related proteins; more concretely PLP, MBP, MOG, and CNP (Krämer-Albers et al., 2007). This data was the first evidence of the possible role that exosomes could be playing in myelination. In a more recent work, it was suggested that Ol-derived exosomes were able to inhibit the differentiation of OPCs (Bakhti et al., 2011). Even though the authors did not demonstrate the mechanism by which Ols regulate OPCs in an inhibitory way, these results reinforce the implication of exosomes in OPC differentiation, an essential step for myelination and remyelination. In a different work, the ability of pregnant mice serum-derived exosomes to promote the trafficking of OPCs into lesions from EAE mice after intravenous administration was shown (Williams et al., 2013) emphasizing the implication of exosomes in myelination related processes.

To analyze the role that EVs play in pathological systems, several models have been used. In a model of white matter infarction in rats, researchers demonstrated that EVs derived from microvascular endothelial cells (MVECs) were taken up by OPCs, inhibiting the apoptosis of OPCs and promoting survival, proliferation and motility of the cells. The authors demonstrate that those EVs contained microRNAs and adhesion molecules which were responsible for the shown effects (Kurachi et al., 2016). Moreover, Mesenchymal Stem Cell-derived exosomes (MSC-Exs) have been shown to promote oligodendrocyte formation and remyelination in a model of subcortical ischemic stroke. After intravenous administration of MSC-Exs, authors

were able to detect higher levels of MOG protein and more myelinated axons. Interestingly, the 2416 proteins detected in the exosomes and described to be involved in brain repair functions were suggested by the authors as mediators of the effect (Otero-Ortega et al., 2017).

Furthermore, a work published in 2014 demonstrated that exosomes from young and environmentally enriched rats significantly increased the myelin content, oligodendrocyte precursor and neuronal stem cell levels and reduced oxidative stress and astrogliosis in demyelinated hippocampal slice cultures (Pusic and Kraig, 2014; Pusic et al., 2016). They also tested the effect of these exosomes *in vivo* by intranasal administration in aged rats, showing positive results in myelin generation. The authors related the exosome-derived pro-remyelination effect to their cargo, suggesting that the presence of miR-219 was responsible for promoting remyelination (Pusic and Kraig, 2014).

Another aspect of demyelinating diseases is that the lack of myelin wrapping axons might, if remyelination does not take place, induce the disruption of the axons and, therefore, neurodegeneration. Neuroprotection is a key factor which might improve patients' outcome and increase their life quality. Regarding to this, mesenchymal stem cells derived EVs were shown to be effective peripheral immunomodulators in models of traumatic brain injury after both intravenous or intraperitoneal administration, decreasing inflammation and increasing neuroprotection, angiogenesis and neurological function, opening therapeutic possibilities in which neuroprotection can be reinforced (Doeppner et al., 2015; Drommelschmidt et al., 2017).

DELIVERY INTO THE CENTRAL NERVOUS SYSTEM

To be able to use EVs as therapeutic biopharmaceuticals for treating MS, it is imperative to ensure that EVs will reach their target cells in the CNS. That can be achieved, for example, by delivering EVs directly to the brain, by using systemic injections, or by administering vesicles via intranasal route. The intranasal route can be efficient for different cell type derived EVs, including T-cell, fibroblast and tumor derived exosomes (Zhuang et al., 2011). This delivery route not only leads to increased brain accumulation of exosomes, but more importantly, it also results in reduced inflammation in EAE animals if exosomes are loaded with therapeutic anti-inflammatory molecules, as was previously mentioned (Zhuang et al., 2011). The latter clearly underlines the potential of EVs for treating MS via the intranasal route, which is further supported by successful experiments conducted in the context of other CNS diseases such as Parkinson's disease (PD). In a mouse model of PD, catalase-loaded macrophage exosomes reached the brain and provided antioxidant-mediated neuroprotection (Haney et al., 2015). Neuroprotection was also induced by curcumin loaded embryonic stem cell exosomes in an ischemia-reperfusion injury model (Kalani et al., 2016). Repeated treatments with curcumin loaded exosomes led to a reduction of inflammation and improved neurological score and restored the expression of several BBB proteins.

However, it appears that EV loading with exogenous cargoes prior to intranasal administration is not always essential for therapeutic effects in the CNS, as recently demonstrated in a status epilepticus mouse model. Unmodified human bone marrow derived MSC-Exs reduced neuron loss and inflammation in the hippocampus of treated mice, which more importantly led to preservation of memory function (Long et al., 2017). These properties of unmodified MSC-Exs for treating CNS disease are particularly interesting and promising for MS. Given the trend toward replacing certain MSC cell therapies with EV based therapies, and the fact that a number of MSC cell therapies have been tested in Phase I/II clinical trials for treating MS as well (Heldring et al., 2015), it is likely that MSC EVs will gain further focus in the short term for targeting MS pathology as well.

In addition to the intranasal administration route, as described above, other local delivery options have shown efficacy for EV based CNS therapies as well. Unilateral direct brain infusion of glioblastoma derived exosomes, pre-loaded with hydrophobic siRNA, led to exosome-dependent bilateral Huntington mRNA silencing in the brain of treated mice (Didiot et al., 2016). Other therapeutic strategies not directly relying on drug delivery can be efficient as well. Intracerebral neuroblastoma exosome administration to an Alzheimer disease mouse model reduced amyloid-β levels in the brain and lowered the associated synaptotoxicity, tapping thus into natural EV-mediated Aβ clearance pathways (Yuyama et al., 2014). Similar effects were observed also when using primary neuron exosomes, the effect being cell type specific as glial exosomes were less efficient in the capture of amyloid-β (Yuyama et al., 2015). This is not surprising as the transport of exosomes to brain parenchyma can be specifically related to the presence of specific surface molecules such as folate receptor α (Grapp et al., 2013) as well as other EV related signatures that can, for example, mediate periphery-brain signaling in inflammation (Balusu et al., 2016).

In many cases, however, systemic rather than local therapeutic EV administration would be preferred for various reasons, including the safety of the treatment administration. Despite the fact that BBB is virtually impermeable to most molecules there is some evidence that unmodified exosomes can enter the brain to some extent (Yang et al., 2015), but brain exposure is significantly increased when using certain brain targeting ligands such as the rabies glycoprotein derived RVG peptide (Wiklander et al., 2015). The brain targeting RVG peptide, even though the precise targeting mechanism has not been fully elucidated, led to increased brain delivery of siRNA when decorated on dendritic cell exosomes (Alvarez-Erviti et al., 2011). Using that strategy, it was possible to lower the levels of Bace1 on both mRNA and protein levels in the brains of wild type mice (Alvarez-Erviti et al., 2011), and in reduced level of α-synuclein mRNA in S129D α-Syn transgenic mice (Cooper et al., 2014).

CHALLENGES AND OPPORTUNITIES

Two characteristic aspects of MS are inflammation and neurodegeneration. The inhibition of inflammation and the promotion of remyelination are postulated as two therapeutic

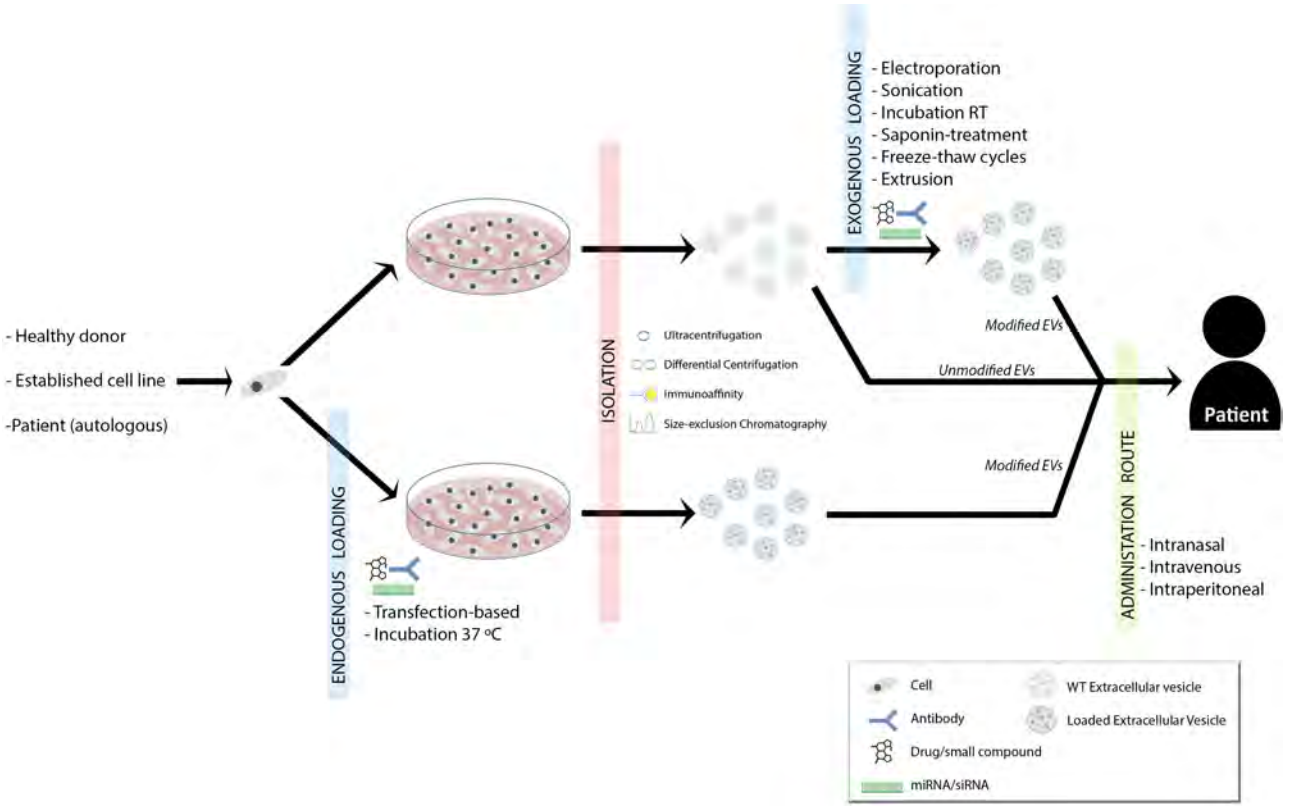

FIGURE 1 | Summary of proposed therapeutic approaches for demyelinating diseases. Established cell lines, donor- or patient-derived cells are isolated and grown. EVs can be loaded with drugs/small compounds, miRNAs/siRNAs and/or surface antibodies, which provide new options in remyelination therapy. The loading can be performed during the cell culture (endogenous loading) or once EVs are isolated (exogenous loading\reviewed in Vader et al., 2016). This might depend on the strategy and purpose of the therapy (Sutaria et al., 2017). Therapeutic EVs can be isolated by ultracentrifugation, differential centrifugation, immunoaffinity or size-exclusion chromatography (Lener et al., 2015). Finally, EVs could be administered to the patient intranasally, intraperitoneally or intravenously and again, this is something that will vary according to the therapeutic strategy.

ways to improve patients' outcome. As it has been widely shown, EVs can play a role in both immunomodulation and remyelination. But, what is the future going to be like with EVs mediated MS therapy? (**Figure 1**).

The First Thing to Consider

The first thing to consider is the source where EVs are isolated from. In this sense established cells lines or cell isolated from the patient or a compatible donor can be used to isolate EVs. Biofluids such as plasma or urine are also an alternative. It is not clear which source is the most efficient and each one has got detractors. In this sense and as explained above, several are the sources that have been used with promising results, opening a wide range of EVs origins to be used. However, we consider that EVs isolated from cell culture might be more reproducible and "easy to manage." In this way, cell therapies derived EVs are suggested as strong candidates as disease treatment. The use of cell-free stem cell-based therapy decreases the risk of cell therapy maintaining the beneficial effect of those cells. As an example, Mesenchymal Stem Cell derived vesicles have been widely studied as therapeutic mediators for several diseases (Review in Börger et al., 2017; Phinney and Pittenger, 2017). We consider that it might be a feasible treatment for MS acting as immunomodulatory agents and tissue repair mediator. In addition to the source, the isolation method is also a relevant aspect to be mentioned, as several methods can lead to different EVs types. Although several effectors has been made in order to standardize isolation techniques, there is still controversy (Gardiner et al., 2016).

A Second Consideration

A second consideration can be the use of non-modified or bioengineered vesicles. The use of non-modified EVs has shown promising results (Pusic and Kraig, 2014; Pusic et al., 2014). In fact, there are several clinical trials recruiting patients in which the ability of allogenic mesenchymal stem cell derived exosomes in acute ischemic stroke or the effect of plasma derived exosomes on cutaneous wound healing will be addressed (NCT02565264, 2015; NCT03384433, 2017). Nevertheless, the modification of the cargo of EVs by bioengineering techniques is an interesting and promising field in EV-mediated therapies and we consider that it might be a more effective treatment method. It has been proved that cells which are genetically modified to overexpress a concrete microRNA, release EVs enriched in that

microRNA (Squadrito et al., 2014). In this sense, microRNAs have shown to be involved in the differentiation of OPCs; more concretely miR-138, miR-219 and miR-338 (Dugas et al., 2010; de Faria et al., 2012; Wang et al., 2017). The enrichment of those microRNAs in the cargo of EVs might induce OPC differentiation and therefore remyelination after demyelination. Vesicles can also be loaded with small compounds and drugs with anti-inflammatory effects. In this sense, curcumin loaded exosomes demonstrated to induce neuroprotection (Kalani et al., 2016). We also propose that nowadays immunomodulatory drugs could also be loaded in exosomes in order to obtain a controlled and direct administration into the CNS. This therapeutic approach is of interest due to the immunological component of MS. Finally, EVs can be modified to express membrane receptors of the target cell, in this way increasing the uptake by the cell and decreasing non-specific bindings (Alvarez-Erviti et al., 2011).

Extracellular vesicles have demonstrated that they are key players in myelin regeneration and the applications that EVs could have in the stimulation of remyelination in pathological states are many. As we have mentioned previously, treatment to induce remyelination is still not available and the use of EVs is becoming a promising and feasible method to immunomodulate, induce myelin restoration, and in this way decreasing neurodegeneration and therefore, increasing patients' outcome. However, even if the implication of EVs in remyelination related processes has been addressed in several works, our knowledge about the therapeutic potential of EVs is just beginning and an exciting future is awaiting us.

AUTHOR CONTRIBUTIONS

IO-Q wrote the sections "Introduction and Therapeutic Potential of EVs for Demyelinating Diseases" and had produced the table and the figure. AA wrote the section "Why Extracellular Vesicles?" IM wrote the section "Delivery into the Central Nervous System." MM-C and DO supervised the work. All authors contributed to the section "Challenges and Opportunities."

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