UNIVERSIDADE D
COIMBRA

Leonor Cunha de Albuquerque Ferreira da Silva

Relatório de Estágio sob orientação da Dra. Susana Sousa e Monografia intitulada "The potential of exosomes as a new therapeutic strategy for glioblastoma" sob orientação da Professora Doutora Maria Teresa da Teixeira Cruz Rosete, referentes à unidade curricular "Estágio", apresentados à Faculdade de Farmácia da Universidade de Coimbra, para apreciação na prestação de provas públicas do Mestrado Integrado em Ciências Farmacêuticas.

Setembro de 2023

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Coimbra, 5 de setembro de 2023.

Leonor Cunha Silva

(Leonor Cunha Silva)

"In science, there are no shortcuts to truth."

- Karl Popper

Agradecimentos

Aos meus pais, por me incentivarem perenemente a primar pela excelência e por investirem de forma contínua na minha educação. Obrigada por me proporcionarem todas as oportunidades, apoio e orientação indispensáveis ao meu sucesso.

A toda a família, por serem o meu suporte e uma fonte de grande inspiração. Obrigada pelo orgulho que demonstram por mim.

À minha notável orientadora, Professora Doutora Maria Teresa Rosete, pelo interesse e disponibilidade constantes, e por todo o auxílio prestado ao longo da elaboração da minha tese. Obrigada pela confiança depositada no meu trabalho e pelas palavras encorajadoras que sempre se encarregou de me transmitir.

Ao Henrique, por fazer deste um percurso mais leve e bonito, por me fazer encarar os períodos mais difíceis com serenidade e por sentir, tão intensamente quanto eu, as minhas conquistas e felicidade. Obrigada.

À Félix, por estar sempre presente e ser um porto de abrigo em todos os momentos. Obrigada.

Às bonitas amizades que a universidade me proporcionou e às amizades de longa data. Obrigada por tornarem esta jornada inesquecível, assinalada por derrotas, mas também inúmeras vitórias. Levo-vos comigo para a vida.

A toda a equipa da Farmácia Sitália, por todo o carinho, boa disposição e preocupação que demonstraram nesta etapa desafiante da minha vida. Obrigada por todos os conhecimentos transmitidos, mas sobretudo por me ensinarem o verdadeiro significado de espírito de equipa e entreajuda.

À Faculdade de Farmácia e aos respetivos docentes. Obrigada por toda a vossa dedicação, sem a qual o meu sucesso e o dos restantes estudantes jamais seria possível.

A Coimbra, por ser a minha cidade-berço, que me viu crescer e me concedeu os melhores 5 anos académicos da minha vida.

A todos, um enorme Obrigada!

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PARTE I

Relatório de Estágio em Farmácia Comunitária

Farmácia Sitália

Sob orientação da Dra. Susana Sousa

Lista de Abreviaturas

AFP - Associação de Farmácias de Portugal

ARSC - Administração Regional de Saúde do Centro

FFUC - Faculdade de Farmácia da Universidade de Coimbra

MICF - Mestrado Integrado em Ciências Farmacêuticas

MNSRM - Medicamentos Não Sujeitos a Receita Médica

MSRM - Medicamentos Sujeitos a Receita Médica

PIM - Preparação Individualizada da Medicação

PNV - Programa Nacional de Vacinação

SNS - Sistema Nacional de Saúde

1. Introdução

De acordo com a Diretiva 2013/ 55/ EU do Parlamento Europeu e do Conselho de 20 de novembro de 2013, a realização de um estágio complementar à formação teórica e prática adquiridas ao longo do curso, é de caráter obrigatório para a obtenção do título de farmacêutico.[1]

Posto isto, o presente relatório foi elaborado no âmbito do Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas (MICF) da Faculdade de Farmácia da Universidade de Coimbra (FFUC), realizado na Farmácia Sitália entre os dias 9 de janeiro e 2 de junho de 2023, sob orientação da Dra. Susana Sousa e colaboração de toda a restante equipa.

Atendendo ao papel preponderante que o farmacêutico comunitário assume na promoção da saúde, prevenção de doenças e prestação de cuidados, o estágio curricular constitui uma oportunidade ideal para aquisição de valências fundamentais à formação de um profissional de saúde qualificado. Assim, durante 5 meses, contactei diretamente com a realidade inerente à profissão farmacêutica, através do exercício de atividades que visam a salvaguarda da saúde pública e o bem-estar do doente.

Este relatório inclui uma análise SWOT (acrónimo para *Strengths, Weaknesses, Opportunities, Threats*), isto é, baseada na identificação de pontos fortes, fraquezas, oportunidades e ameaças respeitantes ao estágio realizado, bem como cinco casos práticos observados no decorrer do mesmo.

2. A Farmácia Comunitária e a sua importância

O farmacêutico comunitário é um profissional de saúde multifacetado, que assume um papel crucial na linha da frente dos cuidados primários de saúde. De acordo com as Boas Práticas em Farmácia Comunitária, "a principal responsabilidade do farmacêutico é para a saúde e o bem-estar do doente e do cidadão em geral, promovendo o direito a um tratamento de qualidade, eficácia e segurança".[2]

A farmácia comunitária foi outrora designada por farmácia de oficina e o farmacêutico considerado como um boticário, dedicando-se à preparação oficinal de medicamentos e produtos farmacêuticos. Contudo, a evolução e adaptação da atividade farmacêutica promoveram a transformação da farmácia num espaço de saúde complexo e focado em suprir as necessidades dos seus utentes.[3] Atualmente, não é considerada apenas como um local de

dispensa de medicamentos, mas também como um local prestador dos mais diversos cuidados e serviços, entre eles o aconselhamento farmacêutico e o acompanhamento farmacoterapêutico do utente. Neste contexto, o farmacêutico comunitário desempenha um papel de extrema relevância, alertando para interações medicamentosas, contraindicações e reações adversas; identificando sinais sugestivos de um diagnóstico precoce de patologias; promovendo o uso racional do medicamento, através da sensibilização da população para a adoção de estilos de vida saudáveis; e selecionando o medicamento mais apropriado para cada indivíduo.

Em diversas circunstâncias, o farmacêutico comunitário é o primeiro profissional de saúde procurado pelo utente, destacando-se uma vez mais o valor por ele assumido na sociedade. Posto isto, é fundamental que se mantenha em constante processo de aprendizagem, de forma a garantir um serviço de excelência à comunidade em geral e ser reconhecido como uma unidade imprescindível para o completo e sustentável funcionamento do Sistema Nacional de Saúde (SNS).

3. A Farmácia Sitália - Enquadramento

Figura 1. Exterior e interior da Farmácia Sitália

A Farmácia Sitália encontra-se localizada na Rua General Humberto Delgado, em Coimbra, sob direção técnica do Dr. João Edgar Rosa dos Reis. Conta também com a colaboração de uma equipa de extrema diligência, composta pelas farmacêuticas Dra. Susana Sousa e Dra. Carla Branco, bem como pela administrativa Célia Simões.

O seu funcionamento decorre de segunda a sexta-feira, entre as 9h e as 19h30, e ao sábado, entre as 9h e as 13h. Nos dias de serviço, definidos anualmente pela Administração Regional de Saúde do Centro (ARSC), a farmácia mantém-se aberta ininterruptamente, desde a hora

de abertura até à hora habitual de encerramento do dia seguinte.[4] Conforme estipulado na legislação vigente, a escala de turnos é publicamente exposta aos utentes no exterior e a palavra "Farmácia" é iluminada durante a noite, quando a mesma se encontra em serviço.[5]

No seu interior, a Farmácia Sitália é composta por duas áreas devidamente separadas: uma correspondente à zona de atendimento, provida de dois postos distintos, e outra provida de um escritório, um gabinete de utente, um armazém, uma zona de receção de encomendas e um laboratório. Os medicamentos estão dispostos primeiramente por forma farmacêutica, de seguida por dosagem e, finalmente, por marca/genérico, respeitando sempre a ordem alfabética. O sistema informático utilizado é o 4DigitalCare®, intuitivo e de elevada praticidade, que permite a gravação de dados extremamente úteis a um atendimento completo, como informações relativas aos utentes (que compõem a designada ficha do utente), informações técnicas e científicas, informações referentes à gestão de encomendas e stocks, entre outras. Garante ainda a privacidade destes dados através de um acesso limitado e sob autorização prévia do respetivo diretor técnico, neste caso o Dr. João Edgar Rosa dos Reis.

A Farmácia Sitália oferece outros serviços além do aconselhamento farmacêutico, como a preparação de alguns manipulados, mediante prescrição médica; a preparação individualizada da medicação (PIM) - para instituições ou utentes; a medição da pressão arterial, da glicémia, do índice de massa corporal e de massa gorda; e a administração de injetáveis ou vacinas passíveis de serem administradas em farmácia comunitária.

Por fim, é de destacar a participação ativa da farmácia em campanhas de grupo e projetos de saúde pública, como a Valormed **(Anexo I)** e o projeto "Seringas Só No Agulhão" **(Anexo II).** A Valormed, iniciada em 1999 com a colaboração de diversos organismos, visa a recolha de embalagens de medicamentos utilizados, ou fora do prazo, e seu posterior tratamento.[6] Por outro lado, o projeto "Seringas Só No Agulhão", criado em 2019 pela Associação de Farmácias de Portugal (AFP), surgiu em resposta à falta de soluções seguras e ecológicas relativas à recolha de seringas e agulhas administradas por doentes diabéticos, ou outros cidadãos que carecem de medicamentos injetáveis.[7] Com a finalidade de sensibilizar os utentes para estas temáticas, dois contentores distintos estão presentes no interior da farmácia **(Anexos I e II).**

4. Análise SWOT

Através de uma análise SWOT - acrónimo anglo-saxónico para *Strengths* (Forças/ Pontos Fortes), *Weaknesses* (Fraquezas/Pontos Fracos), *Opportunities* (Oportunidades) e *Threats* (Ameaças) - pretendo fazer uma avaliação crítica de aspetos do estágio curricular que contribuíram para o meu processo de aprendizagem. Esta análise será realizada através de uma dimensão interna, referente aos Pontos Fortes e Pontos Fracos, e uma dimensão externa, referente às Oportunidades e Ameaças. A dimensão interna abrange os aspetos positivos e negativos da aprendizagem obtida, estando exclusivamente dependente do indivíduo, contrariamente à dimensão externa, que envolve características do ambiente externo não controláveis pelo indivíduo.[8]

4.1. Pontos Fortes (*Strengths*)

4.1.1. Equipa profissional e ambiente de trabalho

Um dos pilares da Farmácia Sitália é, indubitavelmente, a sua equipa profissional de excelência. Foi desde logo percetível que a simpatia, o dinamismo, a capacidade comunicativa e o profissionalismo são valores inerentes a todos os elementos. Fui recebida de braços abertos e fizeram questão de me elucidar acerca de todo o funcionamento e organização da farmácia, facilitando a minha integração inicial. Mostraram-se continuamente disponíveis para o esclarecimento de qualquer dúvida ou questão, bem como para a partilha de novos conhecimentos, promovendo o meu crescimento constante, a nível profissional e pessoal. Depositaram confiança nas minhas capacidades, tendo-me sido dada autonomia nas mais diversas tarefas, entre as quais destaco o atendimento ao balcão, que por norma constituiu um motivo de maior receio. Graças a toda a disponibilidade e boa vontade que me transmitiram nesse e outros momentos, tive oportunidade de aplicar os meus conhecimentos e evoluir no sentido de prestar um serviço de qualidade e ao encontro das necessidades dos utentes, primando pela sua saúde e bem-estar.

Os laços interpessoais criados na farmácia, claramente espelhados numa relação de respeito e amizade entre todos, assim como num espírito de entreajuda, revelaram-se cruciais para uma boa dinâmica de trabalho, motivando-me a querer saber mais.

4.1.2. Organização e gestão da farmácia

A devida arrumação e organização dos medicamentos numa farmácia pode impactar o tempo despendido no atendimento ao utente, pelo que a otimização deste processo é crucial para a

prestação de um serviço célere, permitindo simultaneamente uma melhor gestão de stocks e prazos de validade.

Ao entrar na Farmácia Sitália, a organização dos medicamentos não sujeitos a receita médica (MNSRM) sobressai visivelmente, estando dispostos atrás do balcão de atendimento consoante a sua aplicação, e por ordem alfabética. Separados destes, encontram-se os medicamentos não sujeitos a receita médica de venda exclusiva em farmácia (MNSRM-EF), numa prateleira especificamente destinada à sua disposição. Relativamente aos produtos cosméticos, os mesmos estão destacados por marca comercial, nas prateleiras correspondentes.

No que diz respeito aos medicamentos sujeitos a receita médica (MSRM), estes encontram-se numa área da farmácia interdita ao público e estão organizados primeiramente por forma farmacêutica, de seguida por dosagem e, finalmente, por marca/genérico, respeitando sempre a ordem alfabética. Esta arrumação tem por base a gestão das existências de acordo com o seu prazo de validade, seguindo o modelo FIFO ("*first in, first out*"). Deste modo, os medicamentos que apresentam um prazo de validade mais curto são dispostos à frente dos que apresentam um prazo de validade mais longo, garantindo-se a sua dispensa em primeiro lugar. Existem ainda medicamentos que requerem condições de armazenamento especiais, tal como sucede com produtos farmacêuticos que necessitem de ser conservados no frigorífico, a uma temperatura mais baixa (vacinas, insulinas, alguns colírios, entre outros), e com os psicotrópicos e estupefacientes, que são armazenados em local próprio, dado que a sua cedência se rege por regras próprias e o seu stock envolve um controlo apertado.

No decorrer do meu estágio, compreendi então a relevância inerente à organização de uma farmácia, pois esta representou um fator determinante na minha adaptação inicial ao espaço e, posteriormente, na prestação de um atendimento otimizado, isto é, mais rápido e personalizado. Por um lado, o facto dos produtos de venda livre para determinada necessidade se encontrarem dispostos em conjunto nas prateleiras, permitiu-me avaliar rapidamente a melhor opção disponível para o utente no momento do aconselhamento. Por outro, a disposição intuitiva dos MSRM facilitou a dispensa dos mesmos, ao diminuir o tempo de espera do utente, aquando do seu atendimento.

4.1.3. Prestação de serviços

Entre os diversos serviços farmacêuticos passíveis de serem prestados pelas farmácias, destaco a medição de parâmetros analíticos (pressão arterial, glicémia, colesterol, peso, altura, índice de massa corporal e índice de massa gorda), o aconselhamento farmacêutico, a PIM, a

administração de vacinas e injetáveis, e consultas de acompanhamento farmacoterapêutico, tendo estes sido os serviços que mais presenciei e desempenhei durante o meu estágio.[9] São realizados com a finalidade de acompanhar o estado de saúde dos utentes e garantir o seu bem-estar, através da deteção de efeitos secundários, interações medicamentosas, eventuais erros na administração da medicação e, se necessário, intervenção médica.

A pressão arterial evidenciou-se como o parâmetro analítico mais medido na farmácia, possibilitando a aplicação de conhecimentos adquiridos ao longo do curso, respeitantes quer à maneira correta de realizar esta medição, quer à interpretação dos valores de pressão arterial sistólica/máxima e pressão arterial diastólica/mínima.

Por outro lado, o sistema PIM também merece realce. Tem por objetivo assistir o utente numa melhor gestão da sua medicação, sendo o farmacêutico responsável por organizar os medicamentos em caixas dispensadoras, através das quais é possível verificar posteriormente se houve adesão à terapêutica e cumprimento de horários de toma.[10] Permite ao farmacêutico estabelecer um contacto mais próximo com o utente e consciencializá-lo para a importância de uma boa adesão à terapêutica, mas não deverá ser visualizado como uma solução única para a gestão da medicação, atendendo à dificuldade existente em garantir que o utente cumpre, efetivamente, com as tomas devidas.

Entre os diferentes serviços farmacêuticos anteriormente mencionados, tive também a oportunidade de realizar aconselhamento farmacêutico, inicialmente com o auxílio da equipa da farmácia, e mais tarde de forma autónoma. Pude facilmente compreender que um correto aconselhamento vai muito além de saber o que dizer. É fundamental possuir as capacidades comunicativas, verbais e não verbais, que este requere. Nesse sentido, sublinho o papel da Dra. Susana, da Dra. Carla e do Dr. Edgar, que foram figuras indispensáveis na aquisição dessas competências, demonstrando-me e explicando-me com serenidade como deveria ser feito.

4.1.4. Diversidade de clientes

Para além dos utentes fidelizados que frequentemente recorriam à farmácia para adquirir a sua medicação habitual ou efetuar medição de parâmetros analíticos, outros procuravam um atendimento mais personalizado.

A excelente localização da Farmácia Sitália, perto de cafés, ginásios, escolas, e do centro comercial *Alma Shopping*, atrai novos utentes diariamente com as mais diversas necessidades e exigências. Posto isto, contactei em diferentes ocasiões com produtos cuja existência me era desconhecida, e até mesmo com utentes oriundos de outros países. Esta última situação

constituiu um desafio, não só pela língua, mas principalmente pela ausência em Portugal, de certos medicamentos a que estes clientes estão habituados.

Considero que lidar com utentes tão díspares foi um ponto forte do meu estágio, pois alargou os meus conhecimentos e fomentou as minhas *soft skills*, contribuindo para uma experiência mais enriquecedora.

4.2. Pontos Fracos (*Weaknesses*)

4.2.1. Dermocosmética

A Farmácia Sitália trabalha com marcas específicas dentro da área da dermocosmética, pelo que existem outras que acabam por ser menos representadas, ou até por nem estar dispostas nas prateleiras da farmácia. Isto sucede essencialmente pela preferência que os utentes expressam em adquirir este tipo de produtos em parafarmácias, atendendo ao preço mais apelativo que tende a ser praticado nesses estabelecimentos. Por essa razão, existem produtos cosméticos de marcas não expostas dentro da farmácia que estão presentes em stock apenas em situações mais exclusivas, como é o caso de um pedido efetuado por um utente em particular.

Deste modo, considero que a pequena variedade de artigos na área da dermocosmética se revelou um ponto fraco no decorrer do meu estágio, sobretudo pelo menor contacto que estabeleci com este tipo de produtos e, consequentemente, com o seu aconselhamento, o que culminou numa menor aplicabilidade dos conhecimentos teóricos adquiridos nesta área, ao longo do curso.

4.2.2. Espaço entre balcões de atendimento

O espaço relativo à área de atendimento numa farmácia deve ser profissional, calmo e com boa iluminação. Deve também dispor de uma separação entre os vários balcões de atendimento, de modo a permitir uma comunicação ótima e um atendimento privado com os utentes.[11]

No caso da Farmácia Sitália, o espaço existente entre postos de atendimento é relativamente pequeno, o que senti que dificultou a comunicação com o utente nalgumas circunstâncias, fosse pela falta de privacidade que o mesmo poderia sentir, inibindo-se de expressar certas necessidades, fosse pelo desafio a nível comunicativo em determinados momentos de maior afluência, que prejudicava a compreensão do diálogo utente-farmacêutico.

Posto isto, julgo que uma separação mais evidente entre os balcões de atendimento poderia ser um aspeto a melhorar dentro da farmácia. Daqui resultaria um maior conforto e comodidade aquando do atendimento ao balcão, permitindo ao utente expor os seus problemas sem necessidade de recorrer ao gabinete de atendimento personalizado, que alguns preferem evitar.

4.2.3. Receitas eletrónicas no formato desmaterializado/ Receitas sem papel

O novo modelo de "Desmaterialização Eletrónica da Receita" encontra-se em vigor desde 2015. Apresenta vantagens notórias, como a possibilidade de prescrição de diferentes produtos de saúde (MNSRM e MSRM) num único receituário, contrariamente ao que sucedia no caso de receitas em papel.[12] A receita pode ser enviada ao utente através de uma mensagem de texto para o seu telemóvel (SMS), através de um *e-mail* ao qual se anexa o Guia de Tratamento (contendo informação referente aos medicamentos e produtos de saúde prescritos) e/ou através da impressão do próprio Guia de Tratamento.[13]

Contudo, apesar dos benefícios inerentes a este tipo de receitas, é evidente a adversidade que as mesmas ainda constituem para diversos utentes. Seja para os mais idosos, que desconectados frequentemente do mundo tecnológico, não conseguem aceder às mensagens do telemóvel nem à caixa do *e-mail*, seja para utentes de todas as faixas etárias, que muitas vezes não possuem conhecimento da validade das receitas, ou julgam ter medicação prescrita que, na realidade, já foi dispensada. Apesar da aplicação do SNS facilitar o acesso às receitas do utente e informá-lo acerca do seu estado (dispensado, parcialmente dispensado ou por dispensar), muitas pessoas não têm a aplicação instalada e outras desconhecem mesmo a sua existência.

Deparei-me, não tão raramente quanto seria previsto, com este tipo de situações. Dado que procurava, perante estas circunstâncias, encontrar uma alternativa para aceder à receita do utente em questão, denotei que o meu atendimento acabava por se prolongar como consequência deste contratempo. Tal levou-me a considerar o uso de receitas eletrónicas como um ponto fraco do meu estágio, dificultando a otimização do atendimento nestes momentos.

4.3. Oportunidades (*Opportunities*)

4.3.1. Formação contínua

A formação contínua é uma obrigação profissional, sendo responsabilidade do farmacêutico, como agente de saúde pública, procurar manter os seus conhecimentos atualizados num mundo onde a ciência está em constante evolução. Para tal, é importante que frequente cursos de formação, congressos, simpósios e faça leitura de publicações científicas.[2]

Durante o meu período de estágio, além do contacto direto com a realidade intrínseca à profissão farmacêutica, que me proporcionou muitas novas aprendizagens, fui também incentivada a participar em diversas formações, nomeadamente da Spidifen®, Fluimucil® e PharmaNord-Bioativo®. As mesmas contribuíram para o aprofundar dos meus conhecimentos acerca dos produtos comercializados pelas respetivas marcas, mas também para um maior contacto com outros farmacêuticos e profissionais de saúde, tendo-me sido dada a oportunidade de os ouvir e aprender com as suas partilhas de informação.

4.3.2. Gabinete de atendimento personalizado

O gabinete de atendimento personalizado, onde pude observar e desempenhar a prestação de diferentes serviços farmacêuticos, constituiu uma excelente forma de aquisição de novos conhecimentos no decorrer do meu período de estágio. A administração de medicamentos e de vacinas não incluídas no Programa Nacional de Vacinação (PNV), bem como o seguimento farmacológico, a cessação tabágica, a gestão da terapêutica e o controlo da Hipertensão, terão sido os serviços mais prestados.

Embora não tenha contactado diretamente com todas estas situações, a Dra. Susana fez questão de me chamar ao gabinete, sob autorização prévia do utente, sempre que surgiam novos casos clínicos, contribuindo de modo constante para a minha formação enquanto futura farmacêutica. Verifiquei também que esta foi uma ótima oportunidade para estreitar laços com os utentes, que sentiam nestes momentos um maior à vontade e conforto para abordar determinados tópicos sobre a sua saúde e até mesmo revelar informações necessárias a um melhor acompanhamento farmacoterapêutico.

4.4. Ameaças (*Threats*)

4.4.1. Medicamentos esgotados

Infelizmente, a problemática dos medicamentos esgotados foi uma realidade com a qual me deparei durante o período de estágio. Uma das principais origens deste problema é a exportação paralela de medicamentos, resultante das diferenças de preços entre os mercados nacionais do Espaço Económico Europeu, e que afeta significativamente a aquisição dos mesmos por parte da farmácia.[15] Atendendo ao baixo preço dos medicamentos em Portugal, a reposição de stocks é priorizada em países que praticam preços mais elevados, como é o caso dos países nórdicos.

Assim, fui confrontada frequentemente com situações em que os utentes solicitavam medicamentos esgotados em todos os armazenistas, o que comprometia a continuidade da sua terapêutica. Procurámos, perante estes cenários, encontrar diferentes soluções, entre elas a troca de laboratório, a substituição do respetivo medicamento por um genérico ou de marca, ou a troca da molécula por uma equivalente, o que nem sempre era possível. Neste último caso, era necessário recorrer a dosagens ligeiramente superiores/inferiores, ou mesmo a medicamentos importados.

Se por um lado estes contextos representavam um risco potencialmente grave para doentes com patologias crónicas a realizar terapêuticas continuadas, por outro representavam um grande inconveniente para utentes inflexíveis no que toca à mudança de laboratório, ou à transição de um medicamento de marca para um genérico e vice-versa. Dado que nem sempre o utente é capaz de demonstrar compreensão nestes contextos, lidar com as suas emoções torna-se um desafio para o próprio farmacêutico, que muitas vezes acaba por ser culpabilizado erradamente.

4.4.2. Parafarmácias/locais de venda de MNSRM

Os MNSRM podem ser vendidos fora das farmácias, de acordo com o Decreto de Lei n.º134/2005, de 16 de agosto, desde que se cumpram os requisitos legais estabelecidos.[16] Tal representa uma ameaça para as farmácias, considerando que estes estabelecimentos possuem um poder de compra muito superior, adquirindo produtos em grandes quantidades e, consequentemente, praticando um preço final inferior para o consumidor.

A Farmácia Sitália, em particular, está localizada nas proximidades de um centro comercial, que por sua vez conta com uma parafarmácia e um estabelecimento de venda de produtos

fitoterápicos e suplementos, sendo diretamente afetada. Este fenómeno influencia também o preço dos produtos cosméticos, que tendem a ser mais apelativos nestes locais.

O aconselhamento farmacêutico assume então um papel de extrema relevância neste contexto, dado que poderá mesmo ditar a decisão do consumidor. Como especialista do medicamento, é responsabilidade do farmacêutico saber proporcionar ao seu utente um aconselhamento diferenciado e completo, fazendo-o reconhecer a mais-valia que constituiu para a sua saúde dirigir-se a uma farmácia, em detrimento deste tipo de estabelecimentos.

4.4.3. Sazonalidade do estágio

O meu período de estágio envolveu os meses entre janeiro e maio, evidenciando-se uma necessidade de certos produtos diferente daquela que existe entre os meses de junho e dezembro. Ao presenciar a passagem de duas estações do ano, nomeadamente o inverno e a primavera, lidei sobretudo com afeções típicas destas épocas, como constipações, síndromes gripais, e alergias.

Deste modo, acabei por não ter um contacto tão direto com problemas mais característicos do verão, como queimaduras solares e picadas de insetos, sendo tópicos que reconheço como uma falha na minha aprendizagem.

5. Intervenção Farmacêutica - Casos Práticos

Caso Prático I

Utente do sexo feminino, 25 anos, recorreu à farmácia com queixas de prurido intenso, acompanhado de sensação de queimadura vaginal, dispareunia e disúria ligeira, há cerca de uma semana. Refere ainda a presença de um corrimento esbranquiçado, grumoso e inodoro. Nega a toma de qualquer medicação para alívio dos sintomas e afirma nunca ter sofrido a ocorrência previamente. Perante uma descrição sugestiva de candidíase vaginal *de novo*, e após exclusão de outros problemas de saúde, foi-lhe indicada a aplicação de um anti-fúngico, Gino-Canesten® creme vaginal a 1% (contém 10mg/g de clotrimazol), atendendo à preferência que a utente expressou pelo creme, em detrimento do comprimido (vaginal). Foi-lhe posteriormente explicado que deveria proceder à introdução do aplicador com creme o mais profundamente possível na vagina, uma vez por dia, ao deitar, ao longo de 6 dias consecutivos. A utente foi alertada para a importância de realizar o tratamento até ao fim, de modo a evitar recorrências e resistências por parte do microrganismo, bem como para o facto de não dever utilizar tampões durante a terapêutica, nem 3 dias após o seu término. Por fim, foi-lhe recomendado

efetuar a higiene diária com recurso a água e líquido de higiene íntima com pH próximo de 7, utilizar roupa interior de algodão, e priorizar o uso de preservativo durante o contacto sexual, por forma a evitar disseminar a infeção.[17; 18]

Caso Prático II

Utente do sexo masculino, 40 anos, recorreu à farmácia com queixas de diarreia. Afirmou ter defecado quatro vezes nas últimas 24 horas e pediu um Imodium Rapid® para alívio dos sintomas. Negou ter viajado recentemente e não apresentava sinais febris ou de desidratação, nem outros problemas de saúde. Vómitos, dores abdominais e presença de sangue, pus ou muco nas fezes também não constavam do seu quadro clínico, excluindo-se a necessidade de o reencaminhar para o médico. Tratava-se de um caso de diarreia aguda (3 ou mais dejeções em 24h), pelo que lhe foi indicada a toma de uma solução de rehidratação oral, Dioralyte® (1 carteira em 200ml de água após cada dejeção), pois este contém eletrólitos que atuam impedindo o estado de desidratação, e glucose, que promove a sua absorção. Foram-lhe também recomendadas medidas não farmacológicas, nomeadamente evitar alimentos ricos em gordura ou fibras, doces, leite e refrigerantes, bem como uma adequada ingestão de água. Perante o seu pedido inicial de Imodium Rapid® (antidiarreico obstipante contendo 2mg de cloridrato de loperamida por comprimido), e após confirmação de que o utente se encontrava de baixa em casa, foi-lhe explicado que a diarreia aguda é uma situação autolimitada que não deverá ser interrompida, pois constitui um mecanismo de defesa do organismo, mas sim controlada sintomaticamente através da reposição de fluidos, eletrólitos e alteração da dieta.[19; 20; 21]

Caso Prático III

Adulto jovem, sexo masculino, recorreu à farmácia com queixas de pirose/azia, acompanhada de regurgitação, que se agravava após as refeições e em decúbito. Afirmou que os seus sintomas já se prolongavam há cerca de 10 dias, mas que ainda não tinha feito qualquer tipo de medicação, por considerar que se trataria de algo temporário. Negou a presença de outros sintomas e problemas de saúde, tendo-lhe sido indicada a toma de um antiácido, Kompensan® (contém 340mg de carbonato de di-hidróxido de alumínio e sódio), 1 a 2 comprimidos entre refeições ou ao deitar. Foi-lhe recomendado que chupasse ou mastigasse os comprimidos sem os engolir, e que não excedesse a toma diária de 8 comprimidos. No caso de estar a fazer outras terapêuticas, foi alertado de que deveria respeitar um intervalo de 2 horas entre a toma desses medicamentos e a do antiácido, dado que este pode interferir com a absorção de outros fármacos. Advertimo-lo ainda de que poderia vir a experienciar sintomas de obstipação

durante a administração de Kompensan®, atendendo à presença de alumínio na sua composição. Por fim, no que diz respeito a medidas não farmacológicas, o utente foi aconselhado a evitar alimentos que pudessem despoletar episódios de azia (ácidos, picantes, álcool, café, entre outros); a comer devagar, mastigando bem os alimentos; a fazer várias refeições ligeiras por dia; a evitar deitar-se ou efetuar esforços físicos logo após as refeições; e a dormir com a cabeceira da cama elevada.[22; 23]

Caso Prático IV

Utente do sexo feminino, 65 anos, não fumadora, recorreu à farmácia com queixas de tosse acompanhada de expetoração, que se prolongava há de cerca de duas semanas. Procurou aliviar os sintomas através da toma de um antitússico usado pelo filho, Bissoltusin® pastilhas moles, mas sem sucesso. Perante uma descrição sugestiva de tosse aguda (duração até 3 semanas) e produtiva, a utente foi elucidada de que a utilização de antitússicos como o Bissoltusin® (contendo 10,5mg de bromidrato de dextrometorfano por pastilha) não melhorariam os seus sintomas, dado tratar-se de um MNSRM indicado para o tratamento sintomático de tosse irritativa e seca. Assim, e após confirmação de que a utente não sofria de Diabetes *Mellitus*, foi-lhe recomendada a toma de Grintuss® xarope para adultos, 10ml (2 colheres doseadoras), entre duas a quatro vezes por dia, devendo a última toma ser efetuada antes de dormir. O Grintuss® é maioritariamente composto por mel e complexos moleculares vegetais (resinas, polissacarídeos e flavonóides), podendo ser indicado quer em casos de tosse seca, quer em casos de tosse produtiva. Estas substâncias conferem uma propriedade mucoadesiva, protetora e emoliente ao produto, o que lhe permite atuar, tanto ao nível da inflamação da mucosa através de um efeito barreira, como ao nível do muco graças à sua ação lubrificante e muco-reguladora, que o torna mais fluído e facilmente eliminável. Por fim, a utente foi avisada de que não deveria ingerir água durante os trinta minutos subsequentes à toma de Grintuss®, e que seria expectável notar um aumento da expetoração e tosse após as primeiras administrações, dado que os expectorantes atuam pelo aumento da fluidez e fluxo das secreções.[24; 25]

Caso Prático V

Adolescente, sexo feminino, recorreu à farmácia solicitando ajuda para a possibilidade de gravidez não planeada. Afirmou que a relação sexual tinha ocorrido há cerca de 2-3 dias, mas não sabia precisar o número de horas decorridas desde então. Quando questionada acerca do uso de preservativo, negou a sua utilização, bem como a de qualquer outro método contracetivo. Não existindo certezas de que a relação sexual teria sucedido há menos de 72

horas, e após confirmação de que a utente não tinha recorrido a qualquer tipo de contraceção oral de emergência (COE) durante o presente ciclo menstrual, foi-lhe indicada a toma de EllaOne® (contém um comprimido revestido por película com 30 mg de acetato de ulipristal), a ser administrado assim que possível, dada a sua maior eficácia quanto mais rápida for a toma, e no máximo até 120 horas (5 dias) após a relação sexual desprotegida ou a falha do contracetivo. A utente foi alertada de que deveria repetir a toma da COE em caso de vómitos ou diarreia nas primeiras 3-4 horas, que não deveria utilizar outra COE em simultâneo, e que poderia efetuar a sua toma independentemente da altura do ciclo menstrual, sendo contudo expectável alterações do mesmo. Nomeadamente, no caso de ocorrer um atraso entre 5 a 7 dias, seria recomendável realizar um teste de gravidez. Por fim, advertimo-la para outros possíveis efeitos adversos deste medicamento (náuseas, vómitos, cefaleias, tensão mamária e dores abdominais), informámo-la de que não teria efetividade em relações sexuais subsequentes nem preveniria doenças sexualmente transmissíveis, e foi-lhe fornecido o devido aconselhamento acerca de métodos contracetivos regulares, bem como acerca da sua eficácia e segurança.[26; 27]

6. Considerações Finais

A realização deste estágio curricular, uma etapa final da minha formação, foi essencial para a aplicação de conhecimentos teóricos adquiridos ao longo dos últimos 5 anos. A teoria concedeu-me bases indispensáveis, e a prática permitiu-me compreender a sua relevância na rotina diária de um farmacêutico comunitário.

Ao longo do estágio, procurei adequar a minha postura, nível de linguagem e discurso científico, de modo a garantir uma boa comunicação com o utente e assegurar a sua compreensão. Neste sentido, o estágio constituiu uma oportunidade de me superar e aprender a lidar com as mais diversas personalidades, empenhando-me para conquistar a sua confiança e empatia diariamente.

Para além da dimensão comunicativa e humana inerentes à profissão, manter-se atualizado acerca dos mais diversos tópicos na área da saúde, faz parte do dia a dia do farmacêutico comunitário. Assim, graças aos excelentes ensinamentos transmitidos por toda a equipa farmacêutica, pude também evoluir a nível científico.

Termino esta etapa ciente da importância que um estágio em farmácia comunitária representa no percurso de qualquer estudante do MICF. Quer enquanto futuro agente de saúde pública, quer enquanto futuro especialista do medicamento. E apesar de desafiante, reconheço este

percurso como crucial ao meu crescimento, e uma preparação para o futuro que me aguarda enquanto farmacêutica.

Ao ter a honra de integrar uma equipa de excelência que me acolheu desde o primeiro dia e que procurou, incessantemente, contribuir para o meu sucesso, esta experiência tornou-se progressivamente mais frutífera e gratificante. Dito isto, estou imensamente agradecida a toda a equipa profissional da Farmácia Sitália, por me demonstrarem o que significa, verdadeiramente, ser um Bom Farmacêutico. Muito obrigada!

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8. Anexos

Anexo I. Programa Valormed

Anexo II. Projeto "Seringas Só No Agulhão"

PARTE II

Monografia

"The potential of exosomes as a new therapeutic strategy for glioblastoma"

Sob orientação da Professora Doutora Maria Teresa Rosete

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Abstract

Glioblastoma stands for the most common and aggressive type of brain tumour in adults. The initial symptoms are non-specific, making early diagnosis difficult and allowing tumour progression. It is extremely invasive, rapidly proliferating into other areas of the brain, which explains its short rate of survival. Little is known about its risk factors, and current therapy is still ineffective.

Therefore, efforts are being made to develop novel and effective therapeutic approaches against this type of cancer.

Exosomes are being explored as a promising strategy for conveying and delivering therapeutic cargo to glioblastoma cells, as they can fuse with their membrane and, consequently, serve as delivery systems in this context. Moreover, they have emerged as a superior alternative to liposomes considering their reduced toxicity and enhanced ability to attain higher concentrations in the bloodstream.

Due to their nanoscale size, exosomes present the capacity to cross the blood-brain barrier, which constitutes a major hurdle to most chemotherapeutic drugs used against glioblastoma. They can subsequently inhibit oncogenes, activate tumour suppressor genes, induce immune responses, and control cell growth.

However, despite representing a promising tool for the treatment of glioblastoma, further research and clinical studies are still required to overcome limitations regarding exosome biology, engineering, and clinical applications.

Keywords: glioblastoma, therapy, exosomes, delivery system, blood-brain barrier.

Resumo

O glioblastoma representa o tipo mais comum e agressivo de tumor cerebral em adultos. Os sintomas iniciais são inespecíficos, tornando o diagnóstico precoce difícil e permitindo a progressão do tumor. É extremamente invasivo, proliferando rapidamente para outras áreas do cérebro, o que permite explicar a sua curta taxa de sobrevivência. Pouco se sabe acerca dos seus fatores de risco e a terapêutica atual ainda é ineficaz.

Posto isto, são necessárias novas formas de tratamento e têm sido realizados diversos estudos com o intuito de desenvolver abordagens terapêuticas mais efetivas.

Os exossomas estão a ser estudados como uma estratégia promissora para o transporte e entrega de carga terapêutica, devido à sua capacidade de fusão com a membrana das células do glioblastoma, funcionando como sistemas de entrega neste contexto. Além disso, surgiram como uma melhor alternativa aos lipossomas, atendendo à sua reduzida toxicidade e concentração sérica superior.

Graças ao seu tamanho em nanoescala, os exossomas são capazes de atravessar a barreira hematoencefálica, ultrapassando o principal obstáculo para a maioria dos fármacos citostáticos utilizados. Subsequentemente, podem inibir oncogenes, ativar genes supressores tumorais, induzir respostas imunológicas e controlar o crescimento celular.

Contudo, ainda que os exossomas constituam uma ferramenta promissora para o tratamento do glioblastoma, é necessária uma investigação mais aprofundada, com realização de mais ensaios clínicos, de modo a superar as limitações respeitantes à sua biologia, modificação/preparação e aplicação clínica.

Palavras-chave: glioblastoma, terapêutica, exossomas, sistema de entrega, barreira hemato-encefálica.

List of Abbreviations

AGAP2 – ArfGAP with GTPase Domain, Ankyrin Repeat And PH Domain 2

AKT – Serine/Threonine-Protein Kinase B

BBB – Blood-Brain Barrier

BMSC – Bone Mesenchymal Stem Cells

circRNAs – circular RNAs

CMV – Cytomegalovirus

CNS – Central Nervous System

c(RGDyK) – Cyclo (Arginine-Glycine-DTyrosine-Lysine) Peptide

CSF – Cerebrospinal Fluid

CT – Computed Tomography

DC – Dendritic Cell

DEX – Dendritic Cell-Derived Exosomes

DNA – Deoxyribonucleic Acid

DOX – Doxorubicin

EGFR – Epidermal Growth Factor Receptor

ESCRT – Endosomal Sorting Complexes Required for Transport

ESC – Embryonic Stem Cell

ESE – Early-Sorting Endosome

EV – Extracellular Vesicle

FOXA2 – Forkhead Box A2

FUS – Focused Ultrasound

GBM – Glioblastoma Multiform

GDE – Glioma-Derived Exosome

HER2 – Human Epidermal Growth Factor Receptor 2

HSP – Heat Shock Protein

IARC – International Agency for Research on Cancer

IDH – Isocitrate Dehydrogenase

ILV – Intraluminal Vesicle

iNKT – invariant Natural Killer T

JAG1 – Jagged Canonical Notch Ligand 1

lncRNA – long non-coding RNA

LSE – Late-Sorting Endosome

MAP2K – mitogen-activated protein kinase kinase

MAPK – mitogen-activated protein kinase

MGMT – O-6-methylguanine-DNA methyltransferase

MHC – Major Histocompatibility Complex

miRNA – micro Ribonucleic Acid

MRI – Magnetic Resonance Imaging

MSC – Mesenchymal Stem Cell

mTOR – mammalian Target of Rapamycin

MVB – Multivesicular Body

NF – Neurofibromatosis

NOC – N-nitroso Compound

P13K – Phosphoinositide 3-Kinase

PBP – Polymer-Based Precipitation

PBS – Phosphate-Buffered Saline

PDCD4 – Programmed Cell Death 4

PDGFR – Platelet-Derived Growth Factor Receptor

PEG – Polyethylene Glycol

piRNA – piwi-interacting Ribonucleic Acid

PTEN – Phosphatase and Tensin Homolog

PTX – Paclitaxel

RB – Retinoblastoma

rRNA – ribosomal Ribonucleic Acid

RT – Radiation Therapy

RTK – Receptor Tyrosine Kinase

SEC – Size-Exclusion Chromatography

snRNA – small nuclear Ribonucleic Acid

SPION – Superparamagnetic Iron Oxide Nanoparticle

STK – Serine/Threonine-specific protein kinase

ssRNA – single-stranded Ribonucleic Acid

TAA – Tumour-Associated Antigen

TME – Tumor Microenvironment

TMZ – Temozolomide

TNF – Tumor Necrosis Factor

TP53 – Tumor Protein 53

tRNA – transfer Ribonucleic Acid

UC – Ultracentrifugation

UF – Ultrafiltration

VB – Verbascoside

VEGF – Vascular Endothelial Growth Factor

WHO – World Health Organization

1. Introduction

In the last few years, cancer has progressively become a major health problem, growing worldwide and now being regarded as one of the main global causes of mortality. Additionally, most chemotherapeutic agents are associated with systemic side effects and toxicity in both tumour and healthy cells, which highlights the need for alternative treatments.

Glioblastoma (GBM), recognized as the most common primary brain tumour, is characterized by a median survival of less than two years, having a very poor prognosis.[1] According to the World Health Organization (WHO), GBM is classified as a grade 4 tumour, which denotes its high malignancy.[2] It can affect individuals across different age groups, but occurs mostly in older adults, and it is slightly predominant in males.[3]

Since GBM occurs in less than 10 out of 100 000 people worldwide, it is considered a rare disease.[4] While its exact causes and rising tendency are not fully understood, many potential risk factors have been identified, such as ionizing radiation exposure, chemical exposure, and certain genetic conditions.[5]

The initial presentation of patients with GBM is usually non-specific. Neurologic symptoms gradually develop over a period of days to weeks, the most frequent being headaches, seizures, and focal neurologic symptoms.[6] Generally, diagnosis involves magnetic resonance imaging (MRI), although computed tomography (CT) scans and positron emission tomography (PET) scans may also be conducted.[7] Moreover, given that none of the imaging findings are specific, a biopsy is required to confirm the diagnosis and guide treatment decisions.[8]

On almost every occasion, surgical resection is necessary for these patients, as well as radiation therapy (RT) in combination with chemotherapy (this latter with Temozolomide (TMZ), the most used oral chemotherapy drug in GBM).[1] Despite ongoing efforts to slow tumour growth, control symptoms, and improve the quality of life, the prognosis of GBM remains poor. There is an obvious demand for clinical trials to test novel therapies, including immunotherapies, targeted therapies, and combinatory approaches.

In this regard, exosomes may represent a therapeutic solution for GBM. These nano-sized extracellular vesicles are secreted by diverse cell types, including cancer cells. They have garnered substantial attention due to their involvement in intercellular communication, their ability to carry and transfer a variety of bioactive molecules, and their potential applications as both diagnostic biomarkers and therapeutic agents.[9]

A comprehensive understanding of exosomes encompasses knowledge about their biology, function, use as a drug delivery system, and potential benefits.

Hence, this study aims to explore the current advancements comprising the use of exosomes as novel therapies for GBM in the future.

2. Glioblastoma

The term glioma encompasses a diverse range of primary brain tumours, in which GBM is inserted. Histologically, gliomas closely resemble normal glial cells such as astrocytes, oligodendrocytes, and ependymal cells. However, each type of glioma exhibits a wide-ranging spectrum of aggressive biological behaviour. Diffuse gliomas, the most commonly occurring type of glioma, are subdivided into isocitrate dehydrogenase (IDH)-mutant and IDH-wildtype.[10]

GBM is classified as an IDH-wildtype high-grade glioma, along with grade 3 and 4 IDH-mutant astrocytoma, and grade 3 IDH-mutant, 1p/19q-codeleted oligodendroglioma, and it is considered the most frequent and aggressive malignant primary brain tumour in adults.[11; 12]

GBMs are highly heterogeneous and proliferate invasively, making therapeutic approaches difficult. Their biological traits are mainly driven by three prominent features: proliferation, active invasiveness, and rich angiogenesis, which arise primarily from the dysregulation of signalling pathways.[13]

The process of gliomagenesis, in which mature glial cells convert into a cancerous state through neoplastic transformation, is widely acknowledged as the predominant theory of GBM formation and progression.[14]

According to the clinical history of GBM patients, the histological features and the genetic alterations observed, GBMs can be classified in two different types: primary or *de novo* GBM, and secondary GBM. Primary GBMs, also known as IDH-wildtype, most often arise in elderly patients without precedent lower-grade tumours and are the most aggressive. In contrast, secondary GBMs tend to occur in significantly younger adults and evolve slowly from preexisting lower-grade gliomas (grade 2 or 3), with the presence of IDH1/2 mutations reflecting the lower tumour aggressiveness. Primary GBMs are more prevalent (corresponding to 90% of the cases) than secondary GBMs (10%).[15]

Regarding gene expression profiles, *The Cancer Genome Atlas* network outlines four distinct molecular subtypes of GBM: Neural, Proneural, Classical and Mesenchymal.[16] Each of these

subtypes harbours distinct molecular and genetic abnormalities that initiate tumorigenesis, having different patterns of disease progression and survival outcomes.[17]

2.1. Physiopathology of glioblastoma

Genetic alterations

GBM is characterized by a range of genetic changes that contribute to its formation and growth. Accordingly, *EGFR* mutations are present in approximately 60% of GBMs.[18] *EGFR* is the gene encoding the epidermal growth factor receptor (EGFR), which in turn plays a crucial role in cell division, migration, adhesion, differentiation, and apoptosis. As an oncoprotein, EGFR triggers the EGFR-phosphoinositide 3-kinase (PI3K) pathway, resulting in the sustained activation of this signalling route, which ultimately leads to heightened cellular proliferation and survival of mutated cells.[19; 20]

Additionally, the p53 pathway, essential for deoxyribonucleic acid (DNA) repair, cell cycle arrest and apoptosis, is also altered in almost 90% of GBMs.[17] *TP53* mutations disrupt the normal function of the classic tumour suppressor protein p53 (TP53), contributing to the evasion of cell death and fostering genomic instability in GBM. [21]

Malignant progression of GBM is also associated with the inactivation of the phosphatase and tensin homolog (*PTEN*) tumour suppressor gene, located on the long arm of chromosome 10. Deletions or mutations in *PTEN* are associated with 40% of GBMs.[22] This disease frequently exhibits numerical chromosome alterations, with trisomy 7 (whole chromosome gain) and monosomy 10 (whole chromosome loss) being some of the most prevalent. Since *EGFR* is located on chromosome 7, its gain is closely related to *EGFR* amplification.[23]

The lengthening of telomeres is a crucial factor for the uncontrolled proliferation of tumour cells. For that reason, promoter mutations of the telomerase reverse transcriptase (*TERT*) gene, responsible for maintaining telomere length and preventing cell death, lead to increased telomerase activity, playing a significant role in the development of GBM.[24]

The retinoblastoma (RB) protein, encoded by the *RB1* gene, suppresses cell cycle progression. Likewise, *RB1* is often mutated or inactivated in GBM, resulting in uncontrolled cell proliferation.[25]

In addition, the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene is methylated/silenced in approximately 50% of primary GBM cases. *MGMT* encodes a DNA repair protein responsible for eliminating alkyl groups from guanine-rich areas, the main target

of alkylating agents, countering their therapeutic effect in GBM treatment, and leading to chemotherapy resistance. Hence, the methylation status of this gene may be a valuable biomarker for predicting the response to chemotherapy, considering that patients with *MGMT* promoter methylation tend to display heightened sensitivity to alkylating drugs, namely TMZ, and often experience extended survival. [20; 26]

Altered cell signalling pathways

The above-mentioned genetic alterations in both primary and secondary GBMs, give rise to dysregulated pathways and other molecular changes that should be addressed.

There are two main signalling cascades overactivated in GBMs: PI3K/Akt/mTOR and RAS/RAF/MAPK. Notably, scientific evidence supports the existence of a reciprocal inhibitory crosstalk between these two pathways.[27]

The PIK3/Akt/mTOR signalling pathway controls cell growth and regulates cell survival and proliferation. This pathway is activated by RAS and/or receptor tyrosine kinases (RTKs), such as EGFR, and inhibited by the tumour suppressor protein PTEN, usually responsible for blocking PI3K function. Additionally, serine/threonine-protein kinase B (Akt) and the mammalian target of rapamycin (mTOR) are both categorized as serine/threonine-specific protein kinases (STKs) with pivotal roles in regulating cellular proliferation.[27] Not surprisingly, RTK/PI3K/Akt signalling is altered in approximately 90% of GBMs.[28]

Similarly, the RAS/RAF/MAPK pathway (also referred to as MAPK/ERK pathway) exerts control over fundamental cellular processes, including cell proliferation, differentiation, and survival. Upon activation, the RAS protein initiates a cascade of events by recruiting the RAF family of STKs, such as RAF1. Posteriorly, activated RAF, in turn, triggers the activation of mitogen-activated protein kinase kinase (MEK or MAP2K), which phosphorylates mitogen-activated protein kinase (MAPK). The phosphorylation event ultimately prompts the activation of critical transcription factors involved in cell proliferation and anti-apoptotic genes expression.[29]

Finally, as far as angiogenesis is concerned, GBM is an extremely vascularized tumour, so angiogenic molecules like the vascular endothelial growth factor (VEGF) are prominently present.[30]

Some of the aforementioned pathways and other potential molecular targets of GBM are described in **Table 1.**

2.2. Epidemiology

GBM, a WHO grade 4 neoplasm, is an extremely malignant tumour and comprises 50% of all gliomas. Its incidence varies across different regions and populations, but it is less than 10 per 100 000 people globally.[4] It is also affected by other factors, such as age, sex, race, and ethnicity.[31]

Despite being considered a rare tumour, GBM is overly invasive, exhibiting the highest mortality rate among malignant brain and central nervous system (CNS) tumours, along with a dismal prognosis.[4] Its median overall survival is of only 14 to 15 months, and it has a 1, 2 and 5-year survivals estimated as 28.4%, 11.5% and 3.4%, respectively.[27; 32]

Although it may occur at any age, including childhood, GBM usually affects adults, with the median age of diagnosis ranging between 60 to 70 years, and the peak incidence between 45 and 70. Regarding gender, more cases are reported in men as compared to women.[33; 34]

The increasing incidence of GBM is a growing concern in many countries, but the exact reasons behind it are multifactorial and are still being investigated.[5]

2.3. Risk factors

Most patients diagnosed with high-grade gliomas typically lack a family history of brain tumours or identifiable risk factors. To uncover the underlying genetic and environmental causes of GBM has proven to be a highly challenging task with limited success.[35]

Even so, ionizing radiation stands out as one of the few well-established risk factors for the development of GBM and other high-grade gliomas. Numerous studies conducted on atomic bomb survivors and children exposed to ionizing radiation for medical conditions, have consistently demonstrated its negative impact on health.[36] The time elapsed between exposure to irradiation and the onset of brain tumours can exhibit significant variability, ranging from as short as five years to several decades, depending on a higher or lower irradiation dosage.[6]

On the other hand, while there is speculation about low-frequency electromagnetic field exposure being a potential risk factor for brain cancer, the International Agency for Research on Cancer (IARC) has concluded that data are insufficient to classify it as such.[37]

Cellular telephones emit radiofrequency fields, which have also raised concerns regarding their potential linking to tumours. However, even though WHO/IARC classifies radiofrequency electromagnetic fields as possibly carcinogenic to humans (Group 2B), studies examining the

correlation between cell phone use and glioma incidence rates have produced varying and inconclusive outcomes.[34; 36]

Therefore, there is currently no definitive evidence associating non-ionizing radiation from electromagnetic fields and cell phones to the development of GBM.

Moreover, many studies have been conducted to investigate whether occupational exposures are linked to a greater risk of brain cancer, but the literature still contains several inconsistencies respecting this matter.[38-41] The exposure to environmental agents like vinyl chloride, pesticides, herbicides, petroleum refining, and synthetic rubber manufacturing has been related to the development of gliomas in a very imprecise manner.[6]

Concerning lifestyle and diet, limited conclusive evidence is available regarding their impact on the risk of developing gliomas. N-nitroso compounds (NOCs) are potent animal and human carcinogens.[42] Tobacco smoke, cosmetics, automobile interiors, and cured meats stand for the major exogenous sources of NOCs, and multiple studies have demonstrated a correlation between red or processed meat consumption, and glioma. Conversely, two distinct prospective studies have yielded results that do not support an association between meat intake or dietary NOCs, and the risk of adult glioma, casting doubt on this hypothesis.[43]

Furthermore, there is dearth of strong evidence indicating a correlation between GBM and lifestyle habits like alcohol consumption, cigarette smoking or use of drugs of any kind.[35]

While most gliomas arise spontaneously with no underlying genetic disorder, approximately 1 percent of brain tumours are a consequence of rare inherited syndromes, such as neurofibromatosis type 1 (NF1), *NF2*-related schwannomatosis (NF2), Li-Fraumeni syndrome (LFS), familial polyposis syndrome, tuberous sclerosis, and retinoblastoma.[6; 36]

2.4. Clinical presentation and diagnosis

The initial presentation of GBM often lacks specificity, with signs and symptoms resembling those found in both primary and secondary brain tumours, as well as more common benign neurological conditions. Considering the location and size of the tumour, its presentation may vary greatly. These aspects make suspected cases of GBM hard to identify in primary care.[7]

Patients often experience a gradual progression of neurologic symptoms that evolve over a period of days to weeks, the commonest being: headaches (50% to 60%), usually as an initial symptom and closely linked to substantial mass effect, either from the tumour itself or due to obstruction of the ventricular system; seizures, which are initially observed in 25% of patients and, at a later stage of the disease, in 50%; focal neurologic symptoms such as memory loss,

motor weakness, visual symptoms, language deficit, cognitive and personality changes (10 to 40%).[6; 44] Headaches can exhibit different features depending on the specific location, size and growth rate of the tumour. They are most pronounced upon waking and tend to intensify in frequency or severity over time.[7]

The optimal study for evaluating brain tumours is an MRI with contrast, but in cases where patients have contra-indications to MRI or in resource-limited settings, an initial diagnosis of GBM may include a contrast-enhanced CT scan. However, despite being valuable, the CT head scan is less sensitive. [45]

Moreover, none of the imaging findings are specific and, therefore, a biopsy is required to establish both the type and grade of most primary brain tumours. Immunohistochemistry tests are routinely performed, alongside IDH status assessment, to identify the most common alterations in GBM.[46]

2.5. Therapeutic approaches: current and evolving therapies

The current approach for managing most patients with GBM involves a combined-modality treatment strategy consisting of maximum safe surgical removal of the tumour, followed by RT and concurrent chemotherapy with TMZ.[47] TMZ, used as first-line treatment in GBM, is an oral imidazole tetrazine alkylating agent which exerts its therapeutic effect by inducing DNA methylation.[48]

Another therapeutic approach for managing GBM is the implantation of a biodegradable carmustine wafer, known as Gliadel®. Nevertheless, it is not commonly used due to its efficacy constraints, difficulties related to MRI artifacts after implantation, the potential for oedema, and the possible disruption of the blood-brain barrier (BBB), which may increase the likelihood of CNS infection.[49]

VEGF release is necessary for tumour angiogenesis, which in turn supports GBM growth and progression by increasing vascular permeability, and the volume of blood flow in the surrounding area of the tumour. Bevacizumab, a humanized monoclonal antibody, targets VEGF and leads to peritumoral oedema reduction. However, it can cause some significant adverse effects and has shown no improvement in overall survival when combined with TMZ and RT, the standard therapy. Therefore, it is mainly used for recurrent cases.[50] Pazopanib and Cediranib are alternative oral anti-VEGF agents, but their therapeutic efficacy still requires further investigation.[49]

Supportive medication may also be necessary for GBM patients. The vast majority receive corticosteroids to relieve headache, nausea, and vomiting, since they are able to reduce peritumoral vasogenic oedema and mass effect.[51] Dexamethasone is usually the preferred treatment option due to the absence of mineralocorticoid activity. However, steroids should be administered for the shortest amount of time possible, since their extended use may lead to long-term side effects, such as hyperglycaemia.[7]

Considering the diffuse nature of GBM to eloquent areas of the brain, complete surgical removal of the tumour is not feasible, leading to later disease progression or recurrence. Moreover, the presence of the BBB impedes many drugs of achieving the brain parenchyma.[47]

Overall, the prognosis of GBM is poor mainly due to its heterogeneity, drug resistance, and insufficient brain tumour drug delivery. The survival benefit of TMZ remains at a mere 2,5 months, even though it displays antitumor activity and limited toxicity, and other therapeutic strategies against GBM have failed.[26; 52] Targeting cellular pathways frequently altered in GBM, namely the P13K/Akt/mTOR, p53 and RB pathways, or EGFR overactivation, has not led to improved outcomes. This lack of enhancement may be attributed to several factors, such as redundant compensatory mechanisms within pathways limiting the efficacy of targeting a single pathway; insufficient target coverage partially because the BBB restricts the delivery of therapeutic agents to the brain; poor tolerability and safety profiles of targeted therapies.[53]

Thus, the need for new therapeutic strategies for GBM is unquestionable. Currently, there is a significant endeavour to improve drug delivery to the brain. This encompasses methods such as altering the BBB via ultrasound and heat, and creating advanced nanomedicine designed to enhance drug penetration through the BBB. Improving GBM treatment lies in harnessing drug delivery technologies that can better penetrate the tumour and guarantee a controlled release of therapeutic agents, especially in post-surgery regimens, since these remain the primary treatment approach for GBM.[54] In fact, therapeutic research has predominantly focused on nanocarriers owing to their distinctive capabilities in targeting cell surface-specific molecules and efficiently cross the BBB, therefore delivering the drug specifically to the tumour cells.[55]

There is an imperative need to assess a range of innovative and versatile materials, namely exosomes, hitchhiked nanocarriers and porous materials (silica, silicon, metal organic framework), as all these have already demonstrated their remarkable capability to surmount various biological barriers and may greatly impact the treatment of GBM.[49]

On top of that, considering the inherent heterogeneity of GBM and its capacity to escape and resist single treatments, future approaches should explore the combination of various

intervention modalities. These may encompass physical methods such as ultrasound and heat, chemical strategies involving radiation, chemotherapy, and innovative drug delivery technologies, as well as biological interventions like immunomodulators, exosomes, and cell-based delivery systems. Integrating different methods does not only hold the potential to enhance the effectiveness of GBM treatment, but also of improving the quality of life for patients living with such challenging disease.[49;56]

In this study, we particularly explored the potential of exosomes as a therapeutic tool for GBM.

3. Exosomes

3.1. Definition and function

All cells release extracellular vesicles (EVs), either physiologically or due to acquired abnormalities.[57] EVs can be grouped by size into three distinct categories: exosomes (30-200 nm); microvesicles (100-1000 nm); and apoptotic bodies (1–4 μm).[58]

Microvesicles are directly shed from the cellular membrane, while apoptotic bodies are released by cells experiencing apoptosis, and serve as signals for the engulfment of these dying cells. Exosomes, on the other hand, are formed in the endocytic compartment designated as multivesicular body (MVB) by inward budding and are then released by exocytosis into almost all biological fluids.[59; 60]

Exosomes were first discovered in 1983 by Pan and Johnstone during reticulocyte maturation, but only in 1989 were these vesicle-like structures given the name of exosomes. For years, their potential was undervalued as they were thought to be mere "waste bags" of unwanted cellular products excreted to maintain cellular homeostasis.[61; 62] Nonetheless, recent studies suggest that they play an important role in regulating intercellular communication, enabling donor cells to deliver many exogenous materials, such as proteins, lipids, microRNAs (miRNAs), and mRNAs to recipient cells.[59] Furthermore, they can interact with specific peptides, antibodies, and other biomolecules, changing the phenotype of the cells or working as nanocarriers in anticancer therapy.[63]

Exosomes may present an enormous heterogeneity related to their size, content, functional effect on recipient cells, and cellular origin. This determines not only the amount and type of exosomal content, as well as the impact of exosomes in different target cell types, for example apoptosis induction, cell surviving induction, and immunomodulation.[62]

Due to their reduced immunogenicity, bi-layered lipid structure and ability to communicate with other extracellular environments, thus delivering cargo or altering cell function, exosomes are considered attractive therapeutic vectors for many diseases.[58]

3.2. Biogenesis of exosomes

Originating from the endocytic pathway, exosomes are the result of a process involving double invagination of the plasma membrane. It leads to the formation of MVBs containing intraluminal vesicles (ILVs), which are the forerunners of exosomes. Through MVB fusion with the plasma membrane and subsequent exocytosis, ILVs are ultimately secreted as exosomes.[62] The schematic illustration of exosome formation is represented in **Figure I**.

Briefly, exosome biogenesis comprises four main steps: budding, invagination, MVB formation and secretion.[59] The initial step involves a first invagination of the plasma membrane, along with cell-surface and soluble proteins from the extracellular matrix, leading to the creation of early-sorting endosomes (ESEs). ESEs posteriorly mature into late-sorting endosomes (LSEs), which give rise to MVBs through a second invagination of the plasma membrane (endocytosis of the endosomal limiting membrane). By virtue of this process, MBVs end up containing several ILVs.[62] MBVs may now follow two courses: enter the lysosomal pathway and fuse with autophagosomes or directly with lysosomes, being degraded; or fuse with the plasma membrane, releasing the encapsulated ILVs as exosome entities.[64]

To date, several mechanisms have been proposed to clarify the biogenesis of exosomes. Most of them involve the endosomal sorting complexes required for transport (ESCRT) pathway, which consists of four protein complexes: ESCRT-0, ESCRT-1, ESCRT-2, and ESCRT-3, along with auxiliary proteins such as VTA-1, ALIX, and VPS4.[65] The ESCRT machinery is the main responsible for ILV biogenesis and secretion, as its proteins act synergistically, enabling vesicle budding and cargo sorting in MVBs.[66] Additionally, SNARE proteins and Rab GTPases interact and play an auxiliary role in exosome secretion. The SNARE complex mediates MVB-plasma membrane fusion in different cells and Rab GTPases play a major part in the transport and destiny of these intracellular vesicles.[67; 68] Both Rab27a and Rab27b are accountable for MBV docking on plasma membrane, promoting exosome secretion, but Rab27a influences exosome size, while Rab27b impacts the intracellular distribution of MBVs.[69] Rab11 and Rab35, involved in endosome recycling, also sway the release of exosomes into the extracellular fluid.[70]

Alongside ESCRT-dependent pathways, accumulating evidence suggests that ILV formation may arise from ESCRT-independent processes as well, highlighting the roles of complex lipids and other proteins in exosome generation.[71] For example, tetraspanins, often present in

exosomes, may affect their biogenesis and composition in an ESCRT-independent way. Similarly, lipid rafts (formed by complex lipids) facilitate the formation of intraluminal vesicles.[58]

Apropos to this, the formation of ILVs and exosomes is mediated by a diverse number of mechanisms, the majority of which are yet to be fully understood.

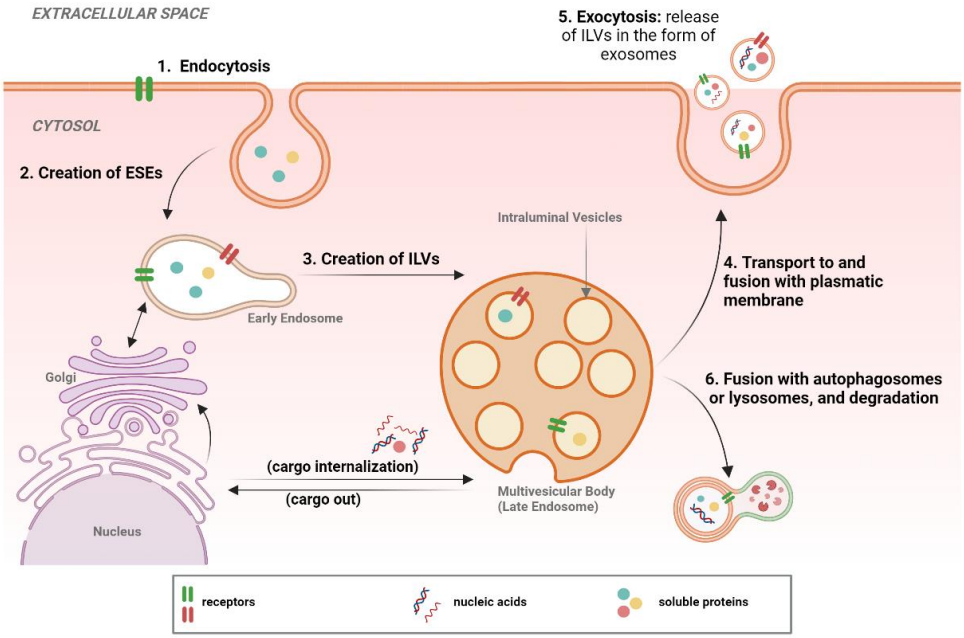

Figure 1. Biogenesis of exosomes (created with BioRender.com). The initial step involves a (1) first invagination of the plasma membrane and subsequent (2) formation of early-sorting endosomes (ESEs), which then (3) mature into late-sorting endosomes (LSEs) / multivesicular bodies (MVBs), rich in intraluminal vesicles (ILVs) derived from ESEs. MVBs may posteriorly (4) fuse with the plasma membrane and release ILVs in the form of exosomes, or (5) enter the lysosomal pathway and be degraded. This figure is adapted from *The exosome journey: from biogenesis to uptake and intracellular signalling*.[58]

3.3. Composition of exosomes

Exosomes contain transmembrane proteins (tetraspanins, antigen-presenting molecules from the major histocompatibility complex (MHC) Class I and II, glycoproteins and adhesion molecules), as well as other types of proteins (heat shock proteins (HSPs), ESCRT proteins and cytoskeletal proteins), lipids, and nucleic acids (DNA and different types of RNA) (**Figure 2**). Interestingly, this cargo varies according to the state of the donor cell (e.g., stressed, activated, or diseased), reflecting its nature and physiological situation.[58;72] Their lipid structure and intraluminal composition are determined by their cell of origin, allowing exosomes to be discerned based on their originating cell.[63]

Exosomes are characterized by an aqueous core and a rigid bilayer membrane rich in lipid components such as cholesterol, ceramides, and sphingomyelin. These are responsible for the morphology of exosomes (stability and structural rigidity), as well as cargo sorting, exosome

secretion and signalling. Considering their composition, exosomes can load both hydrophilic and lipophilic agents.[73]

Since the ESCRT proteins (Alix and TSG-101) are the ones to regulate the exosomal development and MBV shipping, they are expected to be present in all exosomes, regardless of their cell of origin. Likewise, members of the tetraspanin family (CD9, CD63 and CD81), integrin, actin, flotillin, and HSPs should also be found consistently, as they guide exosomes and facilitate their entry and targeting in recipient cells.[74] On the other hand, MHC Class I and II proteins are specific to the donor cell type and take part in T-cell immune-specific responses.[75]

Apart from selected proteins, a set of nucleic acids are abundant in exosomes: DNA, mRNA, miRNA, long non-coding RNA (lncRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and piwi-interacting RNA (piRNA). In particular, miRNAs are one of the most abundant and well-known RNA species in exosomes and take part in their mediated cellular communication.[58] Furthermore, the presence of long RNA species in exosomes such as lncRNAs and circular RNAs (circRNAs) has recently been reported, as well as their influence in many biological processes, including the development of cancer.[76] Thus, studies have investigated the potential of nucleic acids as non-invasive disease diagnostic and prognostic tools.

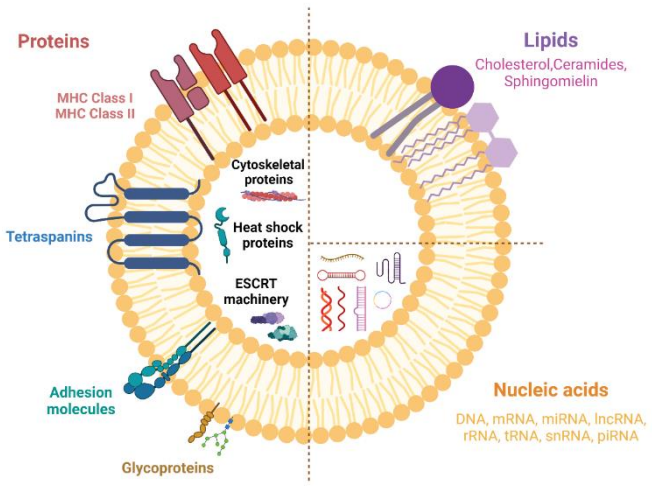

Figure 2. Composition and structure of exosomes (created with BioRender.com). Exosomes are cell-derived membranous structures containing biologically active molecules categorized as proteins, lipids, and nucleic acids.

3.4. Intercellular communication

Exosomes are secreted by most cell types, including erythrocytes, platelets, lymphocytes, dendritic cells (DCs), fibroblasts, adipocytes, macrophages, brain cells, cancer cells and mesenchymal stem cells (MSCs).[77] Hence, they facilitate intercellular communication by

enabling the transfer of exogenous substances from donor to recipient cells, ultimately influencing target cell behaviour and phenotype features.[78] Moreover, via exosome-mediated intercellular communication, they can also play a significant role in immunoregulation through antigen presentation, immune activation, immune suppression, and immune tolerance.[74]

When their surface proteins are recognized by the cell or tissue, exosomes can be internalized in a cell-type-specific manner due to ligand-receptor interactions. The conservation of tropism between donor and recipient cells reflected in the secreted exosomes allows for their uptake by the same recipient cell types, also acting as a targeting specificity.[58] Hence, exosomes exhibit a selective transmission of genetic information to specific recipient cells rather than engaging in random interactions with nearby cells, making them particularly interesting for diagnostic and therapeutic purposes.

When exosomes reach the target cell, they can successfully deliver genetic materials through three primary mechanisms: direct interaction with extracellular receptors (receptor-ligand interaction), direct fusion with the plasma membrane, or internalization via endocytosis, which is the commonest process and enables exosomes to enter as a whole into the cells.[79]

3.5. Therapeutic potential of exosomes

Over the past few years, an increasing number of studies have been addressing the potential of exosomes for diagnosing and treating different pathologies.

Due to their secretion from several types of cells, their presence in almost all biological fluids (breast milk, blood, serum, urine, saliva, amniotic and synovial fluids) and their capacity to regulate complex intracellular pathways, exosomes represent great potential as a therapeutical tool in the control of several diseases, including cardiovascular dysfunctions, neurodegenerative conditions, and cancer.[75]

Exosomes are small and hence can penetrate tissues, diffuse to the blood, cross the BBB and even bypass the P-glycoprotein drug efflux system, reducing drug resistance. As endogenous substances that can be isolated from the patient itself, exosomes possess high biocompatibility, stability, low immunogenicity, and low inherent toxicity, minimizing adverse reactions.[80] Moreover, they have emerged as a superior alternative to liposomes due to their reduced toxicity and enhanced ability to attain higher concentrations in the bloodstream.[81; 82]

They are also highly engineerable, which enables them to deliver a variety of therapeutic materials, such as siRNAs, antisense oligonucleotides, recombinant proteins, immune modulators, and chemotherapeutic agents, via different delivery approaches. [82]

In fact, regarding the theme of cancer, the secreted exosomal miRNAs are more often tumour suppressors, inhibiting cell growth and targeting cells at a long distance. Nonetheless, exosomes may also derive from tumour cells and be involved in the transmission of oncogenic miRNAs, whose upregulated expression promotes tumour progression and metastasis.[60] For this reason, impeding exosome secretion is also viewed as a viable approach for treating cancer.

Considering this, although their role in cancer, cardiovascular diseases, diabetes, and other pathological states has been well documented, it is important to acknowledge that the biological function of exosomes varies according to their biogenesis, cargo packaging and the environment in which they are inserted.[57]

In this work, focus will be given to the use of exosomes as delivering systems for anti-cancer drugs and other molecules, particularly in the field of brain diseases.

4. Exosomes and glioblastoma

By virtue of their multiple inherent characteristics, exosomes are being harnessed as a novel therapeutic answer for many existing diseases, cancer included.

In fact, they can act as a highly efficient biological delivery system, thus assisting the transfer of genetic material between neighbouring or distant cells, owing to their ability to fuse with other cells and capacity to convey nucleic acids, novel drugs, and many other molecules.[83]

When compared to synthetic nanoparticles, exosomes are considered an optimal drug delivery system, as they encompass many exceptional features: remarkable biocompatibility and stability, minimal immunogenicity and toxicity, long half-life circulation, an inherent capability to target specific tissues, and the aptitude to cross biological barriers, like the BBB.[84]

Nonetheless, utilizing exosomes for therapeutic purposes in clinical contexts presents significant challenges, such as limited exosome production and isolation revenue, intricate structure and composition variability, difficulty in drug loading, high costs, potential safety concerns, and suboptimal targeting and delivery efficiency. [83] Furthermore, exosomes may not be able to access certain types of cancer due to the presence of genetic and phenotypic heterogeneity within tumours.

4.1 Exosomes as drug delivery systems in cancer therapy - design and production

Despite being carriers of bioactive cargos, natural exosomes often fall short of achieving the desired therapeutic outcomes. [85] Therefore, they can be modified to carry a precise selection of cargo, including drugs, genetic material, targeting ligands, and tumour antigens, which may be utilized as an anti-cancer vaccine.[63] To do so, exosomes must undertake three indispensable steps, namely: isolation, purification, and incorporation of therapeutic agents.

4.1.1. Isolation and purification of exosomes

Exosomes can be isolated using a variety of methods. The selected method will impact their purity, quantity, and physical-chemical characteristics. For this reason, parameters such as simplicity, celerity, efficiency, scalability, purity, and reliability should be considered at the time of choice. [86]

Among different procedures, ultracentrifugation (UC), size-based isolation techniques, immunoaffinity capture techniques, and polymer-based precipitation (PBP) techniques are the most used.[87] These methods are described in **Table 2**.

UC, which is based on size and density variations, as well as multiple centrifugal forces and rotational speeds, is considered the gold standard for the extraction and separation of exosomes. Firstly, a sequence of ongoing centrifugation at low to moderate speed enables the removal of dead cells, cell debris and large-size EVs. Subsequently, exosomes are rinsed off with a buffer solution like phosphate-buffered saline (PBS) in order to eliminate possible contaminants, and then separated at a higher centrifugal speed. UC is easily implemented, at low cost, and requires minimal reagents and expertise.[88] However, it is time-consuming and may contaminate or damage exosomes.[86]

The size-based isolation techniques include ultrafiltration (UF) and size-exclusion chromatography (SEC). These two methods rely on size differences to separate exosomes from other components and may be combined. [89] UF is said to be a better alternative to UC, offering the advantage of a higher exosome yield and improved isolation efficiency, in a shorter processing time. [90]

On the other hand, capture-based techniques, closely related to immunoaffinity, depend on the utilization of specific antibodies, which selectively bind and capture exosomes by targeting the antigen expressed on their surface. These antibodies may be attached to plate (i.e., ELISA), magnetic beads, resins, and microfluidic devices.[86] This approach enables the isolation of

exosomes with strong specificity, high purity, and high sensitivity, and demands less volume sample than UC.[91] However, the storage conditions of the acquired exosomes are relatively strict and hence, this method is not suitable for large-scale separation.[87]

Lastly, the PBP technique depends on polymers like polyethylene glycol (PEG) or salt solutions such as sodium acetate to precipitate exosomes by reducing their solubility.[90] This procedure is simple, swift, easily scaled up, and does not cause damage to exosomes (in opposition to UC). Nevertheless, it possesses low selectivity, leading to exosome precipitation, but also of other extracellular vesicles, extracellular proteins, and protein aggregates.[92]

4.1.2. Incorporation of therapeutic agents into exosomes

4.1.2.1. Methods for loading cargo into exosomes

Currently, there are two main ways of loading cargo into exosomes: (I) endogenous/in vivo loading (during exosome formation); (II) exogenous/in vitro loading (after exosome isolation) (**Figure 3**). A third category, regarding a fusion method, may also be considered.

Endogenous loading refers to the natural incorporation of therapeutic cargo (drugs, nucleic acids, proteins, and nanoparticles) into exosomes during their formation and maturation within the parental cell, prior to their release in the extracellular environment, thus reflecting the physiological state and functional characteristics of these cells.[93] Only posteriorly are they collected from cells overexpressing the molecule of interest and subsequently isolated. The process itself is straightforward, but the drug loading efficiency cannot be properly controlled and there is a risk of potential damage to the physiological function of membrane proteins.[86]

Transfection and co-incubation are very well-known techniques for in vivo loading.[94] In the transfection method, the parental cells are usually transfected with small RNAs (siRNAs and miRNAs) via a designated vector able to induce their expression, and these small RNAs are further delivered to exosomes by the parental cells.[95] Co-incubation, on the other hand, involves placing certain molecules or therapeutic agents in confined contact with donor cells to obtain and secrete cargo-loaded exosomes. This approach enables the loading of small molecule drugs, as well as nanomaterials.[93] In fact, Pascucci and colleagues incubated MSCs with Paclitaxel (PTX) and collected PTX-loaded exosomes able to induce tumour growth suppression.[96] Similarly, in a study by Wang and colleagues, the incubation of macrophages with curcumin originated curcumin-loaded exosomes with the capacity to cross the BBB for treating Alzheimer disease.[97]

As far as exogenous loading is concerned, the most commonly used methods seem to be incubation and electroporation, while others such as extrusion, sonication, freeze/thaw and saponin-mediated permeabilization are less frequently employed. These techniques involve loading the substances directly into the previously isolated exosomes, and are more effective than endogenous loading, although some aggregation, membrane damage and low yield may occur.[98]

In several studies exploring the potential of exosomes as drug delivery systems, incubation has been widely used as the loading method.[99] In this process, exosomes are incubated with the respective cargo for a certain period, at a specific temperature - either room temperature (22◦C) or body temperature (37◦C). Subsequently, the cargo diffuses into the exosomes by dint of a concentration gradient and cargo-loaded exosomes are finally collected after removing unloaded cargos. [93] Incubation is more suitable for hydrophobic molecules, and despite their limited loading efficiency, this strategy has shown the capacity for loading a diverse range of cargos, such as nucleic acids, small molecular drugs, proteins, and peptides.

In the electroporation method, there is an exposure of the isolated exosomes to short and high-voltage electric pulses, which disturbs their phospholipid bilayer and creates temporary micropores on their membrane. Consequently, cargos are able to migrate into exosomes through these pores.[100] With electroporation, the encapsulation of hydrophilic molecules (e.g. Doxorubicin) is more common, as well as the loading of siRNA and miRNA, since these nucleotides are larger and enable to diffuse into exosomes like small hydrophobic molecules do.[99] To avoid exosome aggregation, it is possible to make use of electroporation buffers like sucrose and trehalose, which strengthen the exosome membrane stability.[101]

Overall, the advantages of exogenous loading include precise control over the type and quantity of cargo loaded into exosomes, the loading of a wide range of therapeutic agents, and easy standardization and reproducibility, making it more amenable for large-scale production.[102]

Lastly, by fusing exosomes and nano-liposomes, it is possible to create liposome-exosome hybrids, which represent composite carriers capable of increasing drug loading efficacy, whilst retaining the function of exosomes. These appear to be more useful for small RNAs and DNA. Nonetheless, a more comprehensive evaluation of such particles is still needed.[103]

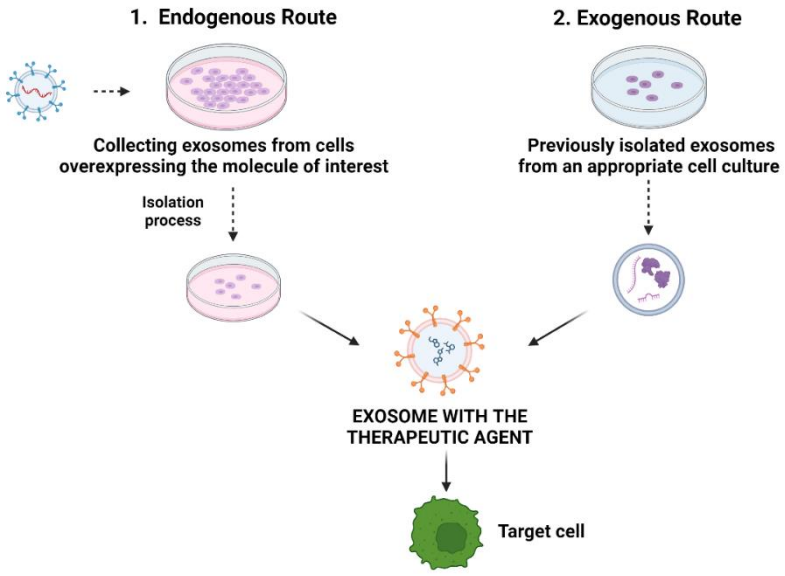

Figure 3. Methods for loading cargo into exosomes (created with BioRender.com). (1) Endogenous loading: involves collecting exosomes from cells overexpressing the molecule of interest and isolating them from the supernatant. (2) Exogenous loading: involves collecting previously isolated exosomes from an appropriate cell culture and loading cargo into them.

4.1.2.2. Types of molecules that can be loaded into exosomes

Exosomes are capable of carrying endogenous materials including lipids, genetic substances (miRNA, siRNA) and proteins (enzymes, cytoskeletal proteins, trans-membrane proteins), but also small molecule drugs like curcumin, dopamine, PTX, doxorubicin (DOX), and gemcitabine.[104]

Among these cargos, miRNAs and siRNAs have attracted significant attention in the field of cancer therapy, as they are able to modulate gene expression and, consequently, affect various cellular processes involved in cancer development and progression.[105] MiRNAs are approximately 22 nanometres in length, single-stranded RNA (ssRNA) molecules that bind to messenger RNA (mRNA) and inhibit protein synthesis by either causing its degradation or suppressing its translation.[106] SiRNAs, on the other hand, are double-stranded non-coding RNAs, usually 20-25 base pairs in length, that target complementary mRNA sequences, thus inducing sequence-specific gene silencing.[107] Exosomes loaded with miRNAs or siRNAs can

target and hinder specific oncogenes or pathways involved in tumour growth, angiogenesis, invasion, and metastasis.[108]

As far as chemotherapeutic drugs are concerned, the bilayer of exosomes allows for the distribution of hydrophobic drugs, while hydrophilic drugs go into their lumen.[109]

The delivery of curcumin, dopamine, PTX and DOX through exosomes has been extensively documented.[110-113] Interestingly, when inserted into exosomes, many of these substances have proven to be more efficient than when acting alone. For instance, exosomal DOX demonstrated faster cellular absorption and increased in vitro effectiveness in various cell lines compared to free DOX and liposomal DOX.[114] Additionally, Pascucci and colleagues reported a significant reduction in the growth of human pancreatic cells owing to the successful loading of PTX in MSC-derived exosomes [96]. Encapsulated curcumin was also described as having an anti-inflammatory activity more than three times stronger than curcumin alone. [115]

In general, exosomal delivery enables higher drug accumulation in target cells, extended blood circulation time and enhanced protection against degradation, thereby improving the effectiveness of small-molecule drugs. [116]

5. Use of exosomes in the treatment of glioblastoma

5.1. The roles of exosomes in glioma tumour microenvironment

A glioma´s tumour microenvironment (TME) is extremely diversified, involving many cancer and non-cancer cells, and is recognized as a strong promoter of glioma growth. [117]

Concomitantly, exosomes also exert numerous functions in cancer progression, playing a dual role in its development: they can either be cancer-promoting or suppressing, according to their cell of origin and bioactive cargo. Additionally, they are essential for facilitating bidirectional communication between tumours and the TME. [118]

In the context of GBM, exosomes have been found to participate in multiple processes within the TME, promoting angiogenesis, facilitating immune evasion, enhancing tumour invasion and migration, and modulating therapy resistance.[119] Glioma-derived exosomes (GDEs) act as mediators of intercellular communication between GBM cells and surrounding cells, such as immune cells, stromal cells, and blood vessels.[120]

GDEs are enriched in non-coding RNAs (i.e., miRNAs), which can be captured by different types of cells, leading to post-transcriptional genetic regulation and behavioural changes associated with tumour growth, invasion, and metastasis. On the other hand, exosomal

ncRNAs can be used for differential diagnosis, monitoring post-surgical glioma progression, and predicting patient responses to personalized therapies.

Among these ncRNAs, miRNAs deserve the spotlight, as they are present in large number in GBM, with both suppressor and oncogenic functions. As a matter of fact, 235 miRNAs are overexpressed in GBM and 95 are downregulated, when compared to normal brain tissue.[121] Exosomal miR-320, miR574-3p and miR-21 correlate with GBM diagnosis. miR-21, usually associated with tumour recurrence or metastasis, has been found in the cerebrospinal fluid (CSF) of 100% of GBM patients, which highlights its potential as a sensitive marker for GBM detection.[122] Lower levels of exosomal miR-151a appear to be connected with resistance to TMZ, indicating a potential biomarker for ineffective chemotherapy treatment.[123]

The hypoxic microenvironment of GBM further contributes to the aggressive behaviour of glioma cells. Hypoxic glioma-derived exosomes promote the polarization of macrophages from an anti-tumour M1 to a pro-tumour M2-like phenotype, enhancing immunosuppression and tumour progression considering M2 macrophages are incapable of killing foreign tumour cells. [124] Hypoxic GBM cell-derived exosomes carry miR-210, so miR-210 could be used as a hypoxia potential biomarker. [125]

Therefore, exosomes transfected with tumour suppressor miRNAs, as well as anti-miRNAs, are promising therapeutic approaches for the treatment of GBM.

Overall, the involvement of exosomes in the glioma TME, namely in intercellular communication, transport of genetic information, and modulation of the immune response, opens avenues for the development of innovative diagnostic and therapeutic strategies.

5.2. Exosome-based therapies for glioblastoma

Exosome-associated treatments against tumours are potentially promising. Indeed, the presence of the BBB (mostly composed by endothelial cells) represents the main hindrance for achieving successful therapies in brain cancer. However, the ability of exosomes to cross the BBB has been demonstrated in a study by Yang and colleagues, where they investigated if siRNA for the *VEGF* gene could be delivered across the BBB in a zebrafish model, by brain endothelial cell-derived exosomes. Not only was it possible to confirm that this type of exosomes could cross the BBB, but the exosomal siRNAs subsequently led to an inhibition of VEGF protein expression in glioblastoma-astrocytoma U-87 MG cells and hindered the aggregation of xenotransplanted cancer cells in the brain of zebrafish.[126]

This example is a perfect demonstration of both the capacity of exosomes to surpass the obstacle of the BBB, and to potentially act as a carrier of different substances for targeted brain delivery.

Therefore, different approaches involving exosomes have been recently developed for cancer therapies, including GBM. These include: (1) using exosomes as gene carriers; (2) engineering exosomes as a vehicle to carry anti-cancer drugs to selective target sites; (3) using exosomes for immunotherapy. [82; 127]

5.2.1. Exosomes as gene carriers

Exosomes have emerged as effective vectors for delivering medicinal and functional RNA in cancer treatments, showcasing their potential as therapeutic agents. Failure to properly regulate miRNAs can heighten the risk of GBM growth and, by targeting specific genes, they can either behave as tumour suppressors or oncogenes. [116]

In gene therapy, since exosomes are less immunogenic than standard transfected agents, they can be loaded with miRNAs or siRNAs and delivered to target cells for different purposes, such as increasing the sensitivity of cells to drugs. [128]

A study from 2013 highlighted the ability of MSCs to release exosomes in significant quantities and incorporate endogenous miRNA mimics into GBM cells, considering that miRNAs can act as oncosuppressors.[129] Therefore, using MSCs acquired from several sources (bone marrow, adipose tissue, placenta, and umbilical cord), Lee and colleagues investigated the delivery of synthetic miRNA to glioma and glioma stem cells through MSC-derived exosomes. They focused on the delivery of miR-124 and miR-145, since GBM cells typically exhibit low expression levels of these miRNAs, and discovered they were successfully transferred by exosomes to GBM cells and could decrease the invasion of glioma cells *in vitro* and *in vivo*, therefore regulating gene expression of recipient cells. [130] Thereafter, treatment with Exo-miR124a effectively suppressed the expansion and clonogenicity of GBM stem cells derived from patients and demonstrated efficacy in treating mice with intracranial GBM stem cell xenografts. MiR-124a also downregulates the expression of Forkhead Box A2 (*FOXA2*), which is responsible for accumulating intracellular lipids that lead to apoptotic cell death if present in abnormal high levels. [131]

In GBM tissues and cells, there is a notable decrease in the levels of miR-512-5p, which targets the Jagged Canonical Notch Ligand 1 (*JAG1*) gene, usually overexpressed in GBM cells. Yan and colleagues transfected bone mesenchymal stem cells (BMSC) with miR-512-5p, and the BMSC-derived exosomal miR-512-5p inhibited GBM cell growth and led to cell cycle arrest by

supressing the expression of *JAG 1*. These results were visible both *in vivo* and *in vitro*, prolonging the lifespan of a mouse model. [132]

The delivery of miR-146b through exosomes showed promising results in the treatment of gliomas by decreasing EGFR and NF-kB proteins *in vitro*, which subsequently resulted in reduced glioma xenograft growth in rat brains. This is crucial data considering that clinical trials performed with EGFR inhibitors have limited success in inducing regression of GBM. [129]

Lei and colleagues reported that isolated exosomes derived from MSCs conveying miR-199a successfully mitigated glioma progression by down-regulating ArfGAP with GTPase domain, Ankyrin Repeat and PH Domain 2 (*AGAP2*) (its overexpression has been related with cancer cell invasion). These MSC-derived exosomes carrying miRNA 199a are internalized by GBM cells, suppressing migration, invasion, and proliferation of U251 cells, which are a type of cell line originated from human GBM. Regarding the obstacle of chemoresistance in GBM, this miR-199a also increases the chemosensitivity to TMZ.[133]

As miRNAs can also behave as "oncogenes", exosomes delivering anti-miRNAs to target cells are a possible therapeutic approach against GBM. For example, miR-9 induces P-glycoprotein expression, an important drug efflux transporter present on GBM cell surface. The administration of anti-miR-9 through MSC-derived exosomes to GBM leads to cell apoptosis and downregulation of P-glycoprotein, thereby reversing multidrug resistance to TMZ. [134; 135]

Furthermore, miR-21 is a well-known tumorigenesis inducer overexpressed in GBM that greatly contributes for its pathogenesis.[116] Exosomal miR21 levels in the CSF typically indicate the likelihood of tumour recurrence or metastasis, having emerged as a potential diagnostic and prognostic biomarker for GBM.[128] On this account, Monfared and colleagues investigated the possibility of down-regulating miR-21 expression in glioma cells, U87-MG and C6, through engineered exosomes packed with a miR-21-sponge construct. Indeed, they were able to inhibit miR-21 while increasing the expression of its targets, Programmed cell death 4 (*PDCD4*) and Reversion Inducing Cysteine Rich Protein with Kazal Motifs (*RECK*), which play pivotal roles in regulating processes related to apoptosis and metastasis. More importantly, these results were later confirmed in a rat model of GBM, where the administration of the manipulated exosomes led to a significant decrease in tumour volume and induced tumour growth deceleration. [136]

These outcomes proposed a possible strategy to block the oncogenic activity of miR-21 and, consequently, reduce the malignant behaviour of GBM cells.

Furthermore, the treatment of tumour-derived exosomes secreted by GBM cells with Verbascoside (VB), an anti-inflammatory and anti-tumour glycosylated phenylpropane compound, promoted the expression of miR-7-5p and its exosomal delivery to recipient GBM cells. Due to the miR-7-5p capacity to downregulate *EGFR* expression and, consequently, to inactivate the EGFR/P13K/Akt pathway, a restraint in GBM cell propagation, invasion, migration, and vessel-like tube formation was observed *in vitro*, and a decline in GBM development and metastasis was observed *in vivo*. [137] Overall, this underlying mechanism is responsible for the inhibitory effect of VB on GBM.

5.2.2. Engineering exosomes as a vehicle to carry anti-cancer drugs to selective target sites

As previously mentioned, exosomes are able to load both hydrophobic drugs within their bilayer, and hydrophilic drugs within their lumen. Considering cancer therapy, they are being largely used as drug carriers mainly because of their stability, tumour targeting, and biocompatibility. [138]

Many researchers have explored the exosomal delivery of PTX, an anti-cancer drug. Regarding GBM, Embryonic Stem Cells (ESCs)-derived exosomes were loaded with PTX and modified with Cyclo (Arginine-Glycine-Aspartic-DTyrosine-Lysine) peptide (c(RGDyK)), a target ligand for cancer chemotherapy. *In vitro* and *in vivo* assays were performed, and the results demonstrated an optimised cancer-cell targeting, together with a strong ability of these exosomes to cross the BBB. As a result, the therapeutic effect of PTX was higher.[139]

DOX is another anti-cancer agent widely studied and currently used to treat various types of tumours. It has already been proven that the cellular uptake of exosomal DOX is faster and its *in vitro* potency is higher, when compared to free DOX and liposomal DOX. [114] Moreover, DOX-loaded exosomes exhibit reduced toxicity and increased intracellular concentration, in contrast to DOX systemic administration alone.[138]

In a relevant study performed by Zhang and colleagues, a system named ENPDOX was developed with the purpose of enhancing the delivery of DOX to the tumour area in patients with GBM and inducing an antitumour immune response. ENPDOX consisted of nanoparticles loaded with DOX, coated with exosomes isolated from mouse brain endothelial bEnd.3 cells (a murine brain endothelial cell line), and these exosomes were able to penetrate the BBB both *in vitro* and *in vivo*. *In vitro*, ENPDOX prompt apoptosis and immunogenic cell death of glioma GL261 cells. Likewise, its systemic administration inhibited GBM growth and promoted apoptosis of GBM cells *in vivo*, leading to a lifespan extension of mice bearing GBM.[140] These

results highlight the potential of ENPDOX as a therapeutic approach for GBM, but is it important to emphasise that additional research is required to assess its suitability for clinical use.

Bai and colleagues conducted a comparison and examination of the therapeutic potential of DOX-loaded exosomes after developing a transportation system using focused ultrasound (FUS) to enhance the targeted delivery of exosomes in glioma therapy. The study revealed that both macrophage-derived and blood-serum-derived exosomes, with the assistance of FUS, effectively delivered DOX into the tumour and suppressed its growth, either *in vitro* or *in vivo*.[141]

Another example in the management of GBM involved the conjugation of MSC-derived exosomes with superparamagnetic iron oxide nanoparticles (SPIONs) to form Ex-SPIONs, which were subsequently loaded with DOX. By virtue of the magnetic characteristics of SPIONs, it was possible to observe the targeted delivery of DOX-loaded Ex-SPIONs in rat brains, along with appropriate drug loading efficiency and stronger cytotoxic effect against tumour cells. [142]

Still regarding DOX and PTX, a recent study conducted by Takur and colleagues revealed that the two aforementioned anti-cancer drugs, which present poor loading efficiency into exosomes, could be more effectively loaded through a developed microfluidic device, Exo-Load. Additionally, autologous exosomes, namely SF7761 stem cell-like derived exosomes, were used to enhance the exosomal delivery of DOX and PTX to SF7761 glioma cells. When compared to U251-GM-derived exosomes, autologous exosomes seemed to be superior for glioma drug targeting. This study revealed that loading anti-cancer agents into exosomes using Exo Load microfluidic device and subsequent autologous uptake of these loaded exosomes by glioma cells, could successfully hinder their proliferation.[143]

Selumetinib is a MAP2K inhibitor, interfering with the MAPK/ERK pathway. Lee and colleagues employed Selumetinib as a prospective therapy for GBM, since it specifically targets *NF1*, which is often found mutated in GBM. Exosomes were derived from U87MG human GBM cells and posteriorly loaded with Selumetinib. They realised that the anticancer effects of Selumetinib-loaded U87MG-derived exosomes (U87-Selu exo) have a specific impact in GBM cells. Furthermore, U87-Selu exo exerted no toxicity in healthy liver and brain cells, indicating that they are a viable therapeutic choice for the treatment of GBM. [144]

5.2.3. Using exosomes for immunotherapy

Currently, exosomes have been receiving attention in the field of immunotherapy for the treatment of GBM. More specifically, the immunotherapeutic potential of tumour cell- and immune cell-derived exosomes in GBM has been aim of research.[145] Taking advantage of their natural intercellular communication skills, exosomes can interact with immune cells and modulate immune responses in the TME.

The activation of immunity primarily relies on the presentation of antigens by exosomes, whereas the inhibition of immune responses mainly depends on the proteins, ligands, and miRNAs they carry.[146] Thereby, one possible approach involves incorporating specific antigens or immune-stimulating molecules into exosomes to promote the activation of immune cells and elicit an anti-tumour immune response.

DCs are the most powerful antigen-presenting cells, activating and stimulating the proliferation of T and B cells.[147] Zitvogel and colleagues showcased that DC-derived exosomes (DEX) loaded with tumour-derived peptides effectively induced a potent T cell response that targeted and eliminated tumours, in a tumour-mice model.[148] In another study by Munich and colleagues, it was demonstrated that DEX can directly induce tumour cell death and activate NK cells because they express tumour necrosis factor (TNF) superfamily ligands (TNF, FasL, and TRAIL) on their surface, which play a pivotal role in triggering apoptosis in tumour cells. [149]

Moreover, given that tumour-derived exosomes have an immunostimulatory effect on anti-cancer DCs, a recent study conducted by Chen and colleagues utilized tumour-derived exosomes as an antigen source for loading DCs, and alpha-galactosylceramide as an adjuvant to activate invariant natural killer T (iNKT) cells. The results revealed enhanced tumour-associated antigen (TAA) presentation by DCs. Consequently, the co-delivery of tumour-derived exosomes with alpha-galactosylceramide-pulsed DCs prompt a strong antitumor immune response in vivo, demonstrating notorious effects in GBM immunotherapy. [148]

All of the aforementioned studies clearly highlight the potential of DEX and tumour-derived exosomes as a promising strategy for cancer immunotherapy.

Besides tumour-derived exosomes, immune cell-derived exosomes have already been experimentally applied in cancer vaccines for immunotherapy since they are capable of modulating the TME and create an anti-tumour microenvironment. [150]

For instance, exosomes derived from NK cells have the potential to enhance cancer vaccines by stimulating pro-inflammatory responses within the TME. Hence, to harness the therapeutic

potential of NK cells in cancer, researchers developed NK-derived exosome mimetics (NK-EM) as a novel approach to immunotherapy. Primarily, with the formation of these nanovesicles, they pretended to address the difficulty of isolating and purifying exosomes obtained in low quantities from mammalian cells. Subsequently, the NK-EM were investigated for their anti-tumour effect, which was confirmed both *in vitro* and *in vivo*, the latter using a xenograft GBM mouse model. This study showed that NK-EM displayed anti-tumour activity and exerted stronger killing effects in cancer cells along with increased apoptosis, when compared to traditional NK cell-derived exosomes. Thus, NK-EM offer a promising tool for cancer immunotherapy, including GBM.[151]

Exosomes are also an ideal candidate for cell-free cancer vaccines due to their high content in tumour antigens, including MHC molecules class I and II.[152] DEX have the capability to indirectly load multiple peptide antigens, such as MHC I and MHC II, which are responsible for a proper antigen presentation to T cells and subsequent CD8+ and CD4+ T cell responses, respectively. [153] Using polyclonal CD4+ T cells together with human epidermal growth factor receptor 2 (HER2)-specific DEX, a new vaccine against breast cancer has been developed, and showed therapeutic efficacy in mice with HER2-specific self-immune tolerance.[154] Despite being a study focused on breast cancer, the concept of using engineered exosomes as vaccines to enhance immune responses could potentially be applied to GBM as well. For example, reports have stated that more than 90% of GBMs express the Cytomegalovirus (CMV).[155] Particularly, the CMV protein pp65 has been identified in glioma cells, but not in the surrounding healthy brain, which makes it a potential tumour-specific immunotherapy target.[156] As a result, researchers have been focused on developing CMV-specific immunotherapies against GBM, such as DC vaccines, considering that these are able to stimulate the patient´s immune system to recognize and target CMV-infected GBM cells.[157] Moreover, long-term survival benefits have been observed in GBM patients receiving CMV pp65-targeted vaccination and the feasibility, safety, and induction of antitumoral immune responses from CMV-specific DCs have been demonstrated.[155] However, conflicting results and controversies regarding the presence of CMV in GBM cells still exist, highlighting the necessity of further research and clinical trials on this matter.[158]

Overall, even though exosome-based vaccines for GBM are promising, more research is required. Clinical trials are underway to assess their immunogenicity, safety, and therapeutic effectiveness in GBM.

6. Future breakthrough directions of exosome carriers in glioblastoma

The use of exosomes in a highly malignant form of brain tumour such as GBM, shows considerable potential for upcoming therapies.

Exosomes play a critical role as intermediaries in the GBM TME, facilitating the intercellular communication between tumour cells and various types of immune cells. They contribute for tumour growth, differentiation, metastasis, and drug resistance.[128] But despite their engagement in the occurrence and development of diseases, exosomes hold great therapeutic value as well.

Exosomes derived from different tissues convey distinct biological components, enabling them to transport and deliver a wide array of chemical and biological molecules, such as drugs, genetic material, and proteins. This versatility endows them with notable advantages for therapeutic applications. Some of these include a higher safety profile; a longer circulating half-life; targeting ability; and enhanced penetration of cell membranes and biological barriers like the BBB, due to their nanoscale size and characteristic surface molecules.[128; 159] In the particular case of brain tumours, exosomes demonstrated the ability to cross the BBB in a genetically modified zebrafish model, while exhibiting strong biocompatibility, minimal immunogenicity, low cytotoxicity, extended circulation time and efficient cellular uptake.[160]

Therefore, some future perspectives concerning the curative potential of exosomes for the treatment of GBM comprise: modifying exosomes to serve as carriers for therapeutic payloads, like anti-cancer drugs or gene-editing tools, enabling targeted and localized treatments [161]; using exosomes for immunotherapy; combining exosome-mediated drug delivery with conventional therapies like chemotherapy and RT to improve treatment efficacy and overcome drug resistance[119]; personalized treatment strategies by carefully examining the exosome molecular profiles of individual patients to address the unique characteristics of their tumours[162]; diagnostic biomarkers, considering exosomes contain a diverse range of biomolecules derived from tumour cells, including nucleic acids and proteins, that can offer valuable insights for early GBM detection, continuous monitoring of its progression, and treatment response prediction.[163]

However, regardless of the tremendous therapeutic potential of exosomes, many challenges need to be surpassed and further investigation is necessary. A major part of the studies focusing on exosome carriers remain at the *in vitro* stage, highlighting the importance of

standardized testing and additional *in vivo* investigation.[164] The intricacies of intercellular communication and the need for a deeper understanding of these mechanisms in brain tumours also pose significant difficulties to the use of exosomes for clinical purposes.

In the criteria pertaining to the manufacturing, purification, storage, stability, loading efficiency, and dosage of exosomes, optimization and improvements are imperative.[86] Engineering exosomes to optimize their interaction with specific targets and enhancing their stability remains a complex task. Extensive research is necessary to counteract the innate homing ability of exosomes and prevent off-target events.[159] Scalability and reproducibility of exosomes production also continue to be a problem for the clinical translation of exosomes and so, refinement of their isolation and purification methods is essential to meet the demands of therapeutic applications.[99] Moreover, given the variability of exosomes in terms of size, composition, and cargo content, it is imperative to define and standardize exosome populations in order to guarantee consistent and reproducible therapeutic outcomes.

To date, even though clinical trials have been conducted to explore exosomes as viable diagnostic and therapeutic candidates, there are no ongoing clinical trials registered on ClinicalTrials.gov that specifically investigate exosomes as drug delivery vehicles for treating GBM. [9] It is clearly necessary to strategize, formulate, coordinate, and carry out clinical trials aimed at validating the effectiveness of exosome-based treatments for brain malignancies.

Undoubtedly, exosomes represent a promising tool for the treatment of GBM. Further research and advancements in exosome biology, engineering and clinical applications are requested to overcome the above-mentioned challenges related to the use of exosomes and pave the way for their successful performance in clinical settings.

In the future, given the expected progress in the cancer research field, exosomes will hopefully become an integral component in the treatment of this life-threatening disease.

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Annexes

Table 1. Potential molecular targets in glioblastoma

	Molecular Targets	Linked mechanism
Tumour-intrinsic pathways	P13k/Akt/*mTOR*; MAPK/ERK; Wnt/β-catenin	Responsible for cell growth, survival, and proliferation
Angiogenic signalling pathways	VEGF; VEGFR	Responsible for promoting the formation of new blood vessels (angiogenesis) to support tumour growth and progression
Immunosuppressive pathways	Immune checkpoint molecules: PD-1; PDL-1; CTLA-4	CTLA-4 binding to CD80/CD86 (expressed on antigen presenting cells) and PD-L1 (expressed on tumour or immune cells) binding to PD-1 (expressed on immune cells) negatively regulate T cell activation, leading to immune suppression
Receptor Tyrosine Kinases (RTKs)	EGFR; PDGFR; MET	RTKs dysregulation contributes to aberrant signalling pathways that promote tumour growth, survival, angiogenesis, and invasion
DNA repair pathways	MGMT	GBM cells often have enhanced DNA repair mechanisms that lead to chemotherapy and radiation therapy resistance
Cancer stem cells' pathways and specific markers	The canonical Notch, Hedgehog and Wnt pathways; CD133, *SOX2*, and NESTIN markers	GBM contains a subpopulation of cancer stem cells, which contribute to tumour initiation, maintenance, and resistance to therapy
Epigenetic modifiers	DNMTs; HDACs	Epigenetic alterations, including DNA methylation and histone modifications, contribute to the development and progression of GBM

CTLA-4, cytotoxic T lymphocyte antigen 4; DNMTs, DNA methyltransferases; EGFR, epidermal growth factor receptor; HDACs, histone deacetylases; MAPK/ERK, mitogen-activated protein kinase/ extracellular signal-regulated kinase; MET, mesenchymal epithelial transition; MGMT, O-6-methylguanine-DNA methyltransferase; PDGFR, platelet-derived growth factor receptor; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Table 2. Exosome isolation and purification techniques

Isolation techniques		*Mechanism of isolation*	*Advantages*	*Disadvantages*
Centrifugation techniques	Ultracentrifugation	Size, Density	Gold standard method; Simple; Low cost; Minimal reagents and expertise required	Low purity; Time consuming; Easy contamination and damage of exosomes
Size-based techniques	Ultrafiltration	Size, Molecular weight	Higher exosome yield, improved isolation efficiency and less time-consuming than Ultracentrifugation; used in large scale	Potential pore blockage; Exosome particle size heterogeneity
	Size-exclusion chromatography	Size, Molecular weight	High purity and reproductivity; non-destructive	Complicated
Capture-based techniques	Immunoaffinity and magnetic beads	Affinity	Rapid; High specificity, purity, and sensitivity; Demands less volume sample than Ultracentrifugation	Strict storage conditions; Not suitable for large-scale separation; High cost
Polymer-based techniques	Commercial Kits	Solubility, surface charge	Simple; Rapid; Non-destructive; High yield; Easily scale up; Commercial kits available	Extremely expensive; co-precipitation of non-exosomal substances, which leads to low-purity exosomes