UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
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Instituto de Biociências de Botucatu - Unesp

CARACTERIZAÇÃO DO TRANSCRITOMA CODIFICADOR DA METÁSTASE CEREBRAL DE CÂNCER DE PULMÃO

VANESSA DAS GRAÇAS PEREIRA DE SOUZA

BOTUCATU – SP

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CANDIDATA: VANESSA DAS GRAÇAS PEREIRA DE SOUZA

ORIENTADORA: PROFª DRª PATRICIA P. REIS

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutora no Programa de Pós-Graduação em Ciências Biológicas (Genética).

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Câmpus de Botucatu

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE VANESSA DAS GRAÇAS PEREIRA DE SOUZA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (GENÉTICA), DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DE BOTUCATU.

Aos 21 dias do mês de fevereiro do ano de 2024, às 09:00 horas, no(a) Unidade de Pesquisa Experimental (UNIPEX), Botucatu, SP., realizou-se a defesa de TESE DE DOUTORADO de VANESSA DAS GRAÇAS PEREIRA DE SOUZA, intitulada **Caracterização do transcritoma codificador da metástase cerebral de câncer de pulmão**. A Comissão Examinadora foi constituida pelos seguintes membros: Profa. Dra. PATRICIA PINTOR DOS REIS (Orientador(a) - Participação Presencial) do(a) Departamento de Cirurgia e Ortopedia / Faculdade de Medicina de Botucatu Unesp, Profa. Dra. ERICA NISHIDA HASIMOTO (Participação Presencial) do(a) Departamento de Cirurgia e Ortopedia / Faculdade de Medicina de Botucatu - Unesp, Profa. Dra. SARAH SANTILONI CURY (Participação Presencial) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu - UNESP, Profa. Dra. LETÍCIA FERRO LEAL (Participação Virtual) do(a) Centro de Pesquisa em Oncologia Molecular / Hospital de Câncer de Barretos, Profa. Dra. MÁRCIA MARIA CHIQUITELLI MARQUES SILVEIRA (Participação Virtual) do(a) Centro de Pesquisa em Oncologia Molecular / Hospital de Câncer de Barretos. Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: APROVADA . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. PATRICIA PINTOR DOS REIS

Dedicatória

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Epígrafe

"Ninguém educa ninguém, ninguém se educa a si mesmo, os homens se educam entre si, mediatizados pelo mundo."

Paulo Freire

Sumário

Resumo

O câncer de pulmão é uma das fontes mais frequentes de metástases cerebrais (MC). Para câncer de pulmão de células não pequenas (CPCNP), cerca de 7 a 10% dos pacientes apresentam MC no momento do diagnóstico, e 20 a 40% dos pacientes desenvolvem MC durante o curso da doença. Apesar das estratégias de tratamento atualmente disponíveis, o prognóstico dos pacientes permanece desfavorável, com uma sobrevida média de 15 meses para pacientes tratados. Diante desse cenário desafiador, torna-se evidente a necessidade de estudos moleculares em larga escala, visando identificar biomarcadores clinicamente aplicáveis para melhorar o desfecho da doença e o prognóstico dos pacientes. Este trabalho oferece uma visão abrangente do estado da arte no estudo das MC abordando eventos-chave na formação da MC, a influência do microambiente tumoral e determinantes moleculares da progressão. Além disso, a integração de dados de sequenciamento de RNA total (RNA-Seq), microarray e sequenciamento de RNA de célula única (scRNA-Seq), juntamente com ferramentas avançadas de bioinformática, foi realizada com o objetivo de identificar os mecanismos subjacentes à formação da MC no adenocarcinoma de pulmão, o subtipo histológico mais incidente de CPCNP. A combinação de RNA-Seq e microarray identificou um conjunto de 20 genes associados a MC do adenocarcinoma pulmonar, principalmente relacionados a vias do sistema imune, destacando a importância do sistema imunológico na formação da MC. Adicionalmente, o uso do scRNA-seq permitiu elucidar o microambiente tumoral da MC e de tumores primários de diferentes estadios de progressão, revelando diferenças na infiltração de células imunológicas. Células T foram identificadas como predominantes em tumores primários, enquanto as microglias predominaram nas MC. A análise de enriquecimento de vias revelou que as microglias apresentam uma marcada upregulação da via de sinalização COX, associada à regulação da resposta inflamatória, indicando um potencial papel da inflamação no desenvolvimento das MC. A infiltração de células supressoras mieloides derivadas de polimorfonucleares (PMN-MDSCs) em MC reforça a importância da infiltração de células imunossupressoras na inflamação crônica associada a essas metástases. A interação entre microglias ativadas, células dendríticas CD163+ CD14+, Th17 e PMN-MDSCs sugere um ambiente inflamado imunossupressor nas MC, com implicações na progressão do tumor. A pesquisa também destaca a interligação entre a sinalização JAK-STAT, interleucina-23 (IL-23) e a produção de interleucina 17 (IL-17), mediadas por microglia ativada, células dendríticas CD163+ CD14+ e Th17, evidenciando uma complexa rede molecular e imunológica na formação das MC. Em resumo, este estudo propõe uma abordagem abrangente, integrando dados de sequenciamento de RNA e ferramentas computacionais para compreender as MC de adenocarcinoma de pulmão. A identificação de biomarcadores, a compreensão das vias moleculares e a análise do microambiente tumoral fornecem *insights* valiosos para o desenvolvimento de terapias mais eficazes e direcionadas, abrindo caminho para avanços significativos no tratamento das metástases cerebrais do câncer de pulmão.

Palavras-chave: Adenocarcinoma; Câncer de pulmão; Microambiente tumoral; Transcriptoma.

Abstract

Lung cancer is one of the most frequent sources of brain metastases (BM). For non-small cell lung cancer (NSCLC), approximately 7 to 10% of patients present with BM at the time of diagnosis, and 20 to 40% of patients develop BM during the course of the disease. Despite currently available treatment strategies, the prognosis for patients remains unfavorable, with an average survival of 15 months for treated patients. Faced with this challenging scenario, the need for large-scale molecular studies becomes evident, aiming to identify clinically applicable biomarkers to improve disease outcomes and patient prognosis. This study provides a comprehensive overview of the state of the art in BM studies, addressing key events in BM formation, the influence of the tumor microenvironment, and molecular determinants of progression. Additionally, the integration of total RNA sequencing (RNA-Seq), microarray, and single-cell RNA sequencing (scRNA-Seq) data, along with advanced bioinformatics tools, was performed to identify the underlying mechanisms of BM formation in lung adenocarcinoma, the most incident histological subtype of NSCLC. The combination of RNA-Seq and microarray identified a set of 20 genes associated with lung adenocarcinoma BM, primarily related to immune system pathways, highlighting the importance of the immune system in BM formation. Furthermore, the use of scRNA-Seq elucidated the tumor microenvironment of BM and primary tumors at different stages of progression, revealing differences in immune cell infiltration. T cells were identified as predominant in primary tumors, while microglia predominated in BM. Pathway enrichment analysis revealed that microglia showed a marked upregulation of the COX signaling pathway, associated with the regulation of the inflammatory response, indicating a potential role of inflammation in BM development. The infiltration of myeloid-derived suppressor cells (PMN-MDSCs) in BM reinforces the importance of immune suppressor cell infiltration in the chronic inflammation associated with these metastases. The interaction between activated microglia, CD163+ CD14+ dendritic cells, Th17, and PMN-MDSCs suggests an immunosuppressive inflammatory environment in BM, with implications for tumor progression. The research also highlights the interconnection between JAK-STAT signaling, interleukin-23 (IL-23), and interleukin-17 (IL-17) production, mediated by activated microglia, CD163+ CD14+ dendritic cells, and Th17, demonstrating a complex molecular and immune network in BM formation. In summary, this study proposes a comprehensive approach, integrating RNA sequencing data and computational tools to understand lung adenocarcinoma BM. The identification of biomarkers, understanding molecular pathways, and analyzing the tumor microenvironment provide valuable insights for the development of more effective and targeted therapies, paving the way for significant advances in the treatment of brain metastases from lung cancer.

Keywords: Adenocarcinoma; Lung cancer; Tumor microenvironment (TME); Transcriptome.

1. Introdução

1.1 Epidemiologia e fatores de risco associados ao desenvolvimento do câncer de pulmão

O câncer de pulmão é a causa mais comum de mortalidade por câncer em ambos os sexos em todo o mundo (SUNG *et al.*, 2021). A mortalidade por câncer de pulmão é maior do que as mortes por câncer de estômago, pâncreas e próstata combinados (SUNG *et al.*, 2021). Aproximadamente 2,1 milhão de novos casos são diagnosticados a cada ano, em todo o mundo, e sua incidência está aumentando, principalmente nos países em desenvolvimento (BRAY *et al.*, 2018; SUNG *et al.*, 2021). No Brasil, os dados de incidência estimam mais de 32.560 novos casos para cada ano do triênio de 2023 a 2025, a maioria (~ 18.020) em homens, de acordo com o Instituto Nacional do Câncer (INCA, 2023).

O câncer de pulmão é classificado em dois tipos histológicos principais; o câncer de pulmão de células não pequenas (CPCNP, da sigla, em inglês, NSCLC, *Non-Small Cell Lung Cancer*) e câncer de pulmão de células pequenas (CPCP, da sigla, em inglês, SCLC, *Small-Cell Lung Cancer*). Estes carcinomas diferem histologicamente e molecularmente, mostrando mutações e alterações moleculares específicas de cada subtipo. O CPCNP é responsável pela maioria (~85%) dos casos de câncer de pulmão (INAMURA, 2017) e é classificado em diferentes subtipos histológicos, sendo os mais comuns o adenocarcinoma (AD) (~40% dos cânceres de pulmão), carcinoma de células escamosas (CCE) (~25%) e carcinoma de grandes células (~10%) (INAMURA, 2017).

O tabagismo é um dos principais fatores de risco associados ao desenvolvimento do câncer de pulmão. Mais de 90% dos casos estão associados à exposição crônica ao tabaco (HERBST; MORGENSZTERN; BOSHOFF, 2018). Considerando que cerca de metade dos novos pacientes com câncer de pulmão são ex-fumantes, ou indivíduos que pararam de fumar há mais de 10 anos, o câncer de pulmão continuará sendo um grave problema de saúde por muitos anos em vários países, incluindo o Brasil. Além disso, o câncer de pulmão também ocorre em indivíduos que nunca fumaram, muitas vezes devido à exposição passiva aos carcinógenos do tabaco e poluentes ambientais; esta doença é considerada uma doença clínica e molecular distinta em comparação ao câncer de pulmão que surge em fumantes (KORPANTY *et al.*, 2018; SUN; SCHILLER; GAZDAR, 2007).

A incidência de câncer de pulmão entre nunca fumantes apresentou aumento nas últimas décadas. Em Taiwan por exemplo, mais de metade dos casos de câncer de pulmão ocorrem em indivíduos que nunca fumaram (CHIEN *et al.*, 2020). Nos Estados Unidos estima-se que 10% a 15% dos casos de câncer de pulmão ocorrem em nunca fumantes (OFFICE OF THE SURGEON GENERAL (US); OFFICE ON SMOKING AND HEALTH (US), 2004). No Brasil, apesar das políticas de saúde pública terem levado ao declínio do consumo de tabaco nos últimos anos, o câncer de pulmão – sem considerar os tumores de pele não melanoma – é o quarto tipo de câncer mais incidente entre mulheres (14.540) e o terceiro entre homens (18.020); e a segunda causa de morte por câncer no país em homens e mulheres (INCA, 2023). Até 2040, a incidência e mortalidade por câncer de pulmão, no Brasil, são projetadas com aumento para mais de 73 mil casos novos com aproximadamente 65 mil óbitos relacionados à doença (FERLAY *et al*., 2020).

As estratégias terapêuticas para o câncer de pulmão apresentaram avanços substanciais nos últimos anos e incluem ressecção cirúrgica, quimioterapia, radioterapia, terapêuticas com alvos moleculares e imunoterapias (MITHOOWANI; FEBBRARO, 2022). Em geral, pacientes com estadiamento inicial são submetidos à cirurgia, enquanto os pacientes com doença localmente avançada ou metastática são submetidos a terapias sistêmicas (MITHOOWANI; FEBBRARO, 2022). Apesar das opções terapêuticas, o câncer de pulmão ainda tem um prognóstico ruim com sobrevida em 5 anos de 7 a 26% (LU *et al.*, 2019). ***Uma das principais razões para o prognóstico desfavorável reside no diagnóstico tardio da doença, devido à inespecificidade dos sintomas principalmente nos estágios iniciais de desenvolvimento da doença*** (MIRANDA-FILHO *et al.*, 2021)***. Aproximadamente 79% dos pacientes com câncer de pulmão apresentam doença localmente avançada ou metastática (estadios III e IV, respectivamente), reduzindo as possibilidades terapêuticas e, consequentemente, a sobrevida dos pacientes*** (BADE; DELA CRUZ, 2020). Aproximadamente 47% dos pacientes com CPCNP apresentam metástases à distância no momento do diagnóstico, sendo osso, pulmão e cérebro os sítios metastáticos mais comuns (BARTA; POWELL; WISNIVESKY, 2019).

1.2 A metástase cerebral é uma das principais complicações clínicas no CPCNP

A metástase cerebral (MC) é responsável pela maioria dos tumores do sistema nervoso central (SNC), sendo observada em até 40% dos pacientes com diferentes tipos de câncer

(MORAVAN *et al.*, 2020; WANLEENUWAT; IWANOWSKI, 2020). A MC é ~10 vezes mais comum do que os tumores primários que afetam o SNC (MORAVAN *et al.*, 2020; WANLEENUWAT; IWANOWSKI, 2020).

Os cânceres mais comuns com metástase para o cérebro são pulmão, mama, melanoma, rim e cólon (NAYAK; LEE; WEN, 2012). De acordo com os dados do Programa de Vigilância, Epidemiologia e Resultados Finais (da sigla, em inglês, SEER, *Surveillance, Epidemiology, and End Results*), pacientes com CPCNP e CPCP têm as maiores taxas de metástases cerebrais ao diagnóstico (CAGNEY *et al.*, 2017). Aproximadamente 20% dos pacientes com CPCNP apresentam MC no momento do diagnóstico, e entre 20% e 40% desenvolvem MC ao longo da progressão da doença (ACHROL *et al.*, 2019). Além disso, o câncer de pulmão é o tumor primário mais frequentemente associado ao desenvolvimento de MC, independentemente do sexo do paciente, afetando entre 20% e 56% dos casos (INAMURA, 2017). No entanto, é provável que esses números estejam subestimados devido à ausência de recomendação para a realização de ressonância magnética cerebral de rotina em pacientes assintomáticos do ponto de vista neurológico. Adicionalmente, embora estudos tenham reportado a detecção de MC no momento do diagnóstico inicial, não existem dados precisos sobre os locais subsequentes de envolvimento metastático ao longo do curso da doença (ACHROL *et al.*, 2019).

Na maioria dos casos, as MC se manifestam como múltiplas lesões, embora aproximadamente um terço dos pacientes apresente lesões únicas (ACHROL *et al.*, 2019). A presença de MC geralmente está associada ao surgimento de diversos sintomas e sinais clínicos que levam o paciente não diagnosticado a buscar atendimento médico inicial. Estes sintomas podem ser debilitantes e incluem cefaleia, vômitos ou náuseas, paralisia, bem como alterações mentais como problemas de memória, convulsões e tonturas (FERLAY *et al.*, 2010; KORPANTY *et al.*, 2018). Nos últimos anos, observou-se um aumento na prevalência de MC, o que pode ser atribuído a avanços no diagnóstico por imagem cerebral, permitindo a detecção da doença subclínica, assim como às novas opções de tratamento e controle da doença sistêmica extracraniana, o que prolonga a sobrevida dos pacientes e, por conseguinte, permite a disseminação do câncer para o cérebro (FERLAY *et al.*, 2020; LU *et al.*, 2019).

O risco de desenvolver MC em pacientes com câncer de pulmão depende de uma variedade de fatores, incluindo a histologia do tumor (adenocarcinoma ou carcinoma de células escamosas), o grau de malignidade, o envolvimento nodal positivo e a idade jovem (WAQAR *et al.*, 2018). ***Os pacientes com adenocarcinoma constituem mais de 50% de todos os casos***

de carcinoma de CPCNP e exibem uma incidência mais elevada desse tipo de câncer em comparação com aqueles que possuem outros subtipos histológicos (HUBBS *et al.*, 2010; SHI *et al.*, 2006; WAQAR *et al.*, 2018)**.** Além disso, gênero e etnia também estão associados ao desenvolvimento de MC no câncer de pulmão. Embora o valor preditivo do gênero para MC possa permanecer controverso, estudos indicam que as mulheres têm o dobro da probabilidade de desenvolver MC após o diagnóstico do câncer de pulmão primário em comparação com os homens (DING *et al.*, 2012; JACOT *et al.*, 2001). Afro-americanos com câncer de pulmão também demonstraram uma incidência maior de MC do que outros grupos étnicos que possuem o mesmo tipo de câncer (BARNHOLTZ-SLOAN *et al.*, 2004).

A maior incidência de MC em pessoas negras, em comparação com outros grupos raciais, pode ser atribuída a vários fatores, incluindo disparidades no acesso e tratamento de cuidados de saúde. Pesquisas demonstraram que pacientes negros e hispânicos têm menos probabilidade de receber certos tratamentos, como a radiocirurgia estereotáxica para MC, e têm maior probabilidade de enfrentar disparidades no sistema de saúde, incluindo atrasos no diagnóstico e tratamento. Além disso, fatores socioeconômicos, como viver em bairros de baixos rendimentos sem acesso a médicos de cuidados primários e cobertura de seguro de saúde, podem contribuir para os desafios enfrentados pelos indivíduos negros na obtenção de cuidados oportunos e adequados para a MC (AKINYEMIJU *et al.*, 2018; MCCRAY *et al.*, 2023; WILCOX; BOIRE, 2020).

O prognóstico dos pacientes com MC é extremamente ruim, atingindo 12 meses em geral e 15 meses em pacientes com adenocarcinoma pulmonar (SPERDUTO *et al.*, 2020). As MC afetam significativamente o curso clínico da doença em pacientes com malignidade sistêmica; aproximadamente 90% dos pacientes apresentam sintomas neurológicos na apresentação da doença, frequentemente causando profundo prejuízo na cognição, qualidade de vida e status de desempenho (ACHROL *et al.*, 2019).

Embora não sejam o foco central desta pesquisa, as metástases leptomeníngeas formam um subconjunto das metástases cerebrais, manifestando-se no revestimento do cérebro, da coluna ou no líquido cefalorraquidiano (LCR), podendo ocorrer com ou sem metástases no parênquima cerebral. Originárias de células tumorais que se infiltram no LCR por meio de disseminação hematogênica, as metástases leptomeníngeas representam uma complicação grave frequentemente associada a um prognóstico desfavorável (BÖNIG *et al.*, 2019). A incidência de metástases leptomeníngeas varia de 9-25% em cânceres de pulmão, ocorrendo

mais frequentemente em pacientes com CPCP (LAMBA; WEN; AIZER, 2021; NGUYEN *et al.*, 2023). No entanto, essa incidência tem aumentado em subgrupos de pacientes com mutações específicas, devido aos resultados mais promissores de novas terapias moleculares.

Atualmente, as estratégias terapêuticas direcionadas às MC incluem radiocirurgia estereotáxica, radioterapia de todo o cérebro, ressecção cirúrgica, terapia térmica intersticial a laser, quimioterapia citotóxica sistêmica, terapia direcionada e imunoterapia baseada em inibidores de checkpoints imunológicos (MORAVAN *et al.*, 2020). No entanto, apesar dos tratamentos multimodais e dos avanços nas terapias direcionadas, na prática clínica, o tratamento da MC ainda é paliativo e centra-se principalmente em cirurgia e radioterapia, uma vez que o acesso de drogas ao sistema nervoso central (SNC) é limitado devido à permeabilidade altamente seletiva da barreira hematoencefálica (da sigla, em inglês, BBB, *blood–brain barrier*) (ARVANITIS; FERRARO; JAIN, 2020; WANLEENUWAT; IWANOWSKI, 2020). Além das limitações terapêuticas, o manejo da MC apresenta outros desafios, incluindo os efeitos adversos associados aos tratamentos neurocirúrgicos ou radioterápicos. A radioterapia de todo o cérebro (da sigla, em inglês, WBRT, *whole brain radiation therapy*) pode resultar em efeitos adversos agudos, como perda de memória, confusão e leucoencefalopatia (ENE; FERGUSON, 2022). Esses desafios são agravados pelo fato de as MC normalmente ocorrerem no contexto de câncer avançado, tradicionalmente com um prognóstico desfavorável (ENE; FERGUSON, 2022).

Nos últimos anos, os avanços nas tecnologias de sequenciamento têm proporcionado uma compreensão mais aprofundada das vias moleculares subjacentes à formação de MC no câncer de pulmão. A terapia direcionada tem emergido como uma alternativa promissora para o tratamento (ACHROL *et al.*, 2019; FECCI *et al.*, 2019; MORAVAN *et al.*, 2020). Entretanto, a maioria dos estudos avalia as alterações moleculares em tumores primários para prever o desenvolvimento de metástases, o que frequentemente explica as falhas das terapias-alvo no tratamento da MC devido à divergência molecular observada entre a MC e o tumor primário (PREUSSER *et al.*, 2015). É reconhecido que as MC apresentam alterações condutoras potencialmente informativas do ponto de vista clínico (BRASTIANOS *et al.*, 2015). Portanto, estudos moleculares em larga escala, concentrados nas MC, representam uma estratégia promissora para compreender os mecanismos que conduzem à formação das MC. Além disso, os resultados desses estudos certamente serão úteis para o desenvolvimento de estratégias terapêuticas mais eficazes com biodisponibilidade além da barreira hematoencefálica.

1.3 A formação de metástase cerebral é um processo complexo

O desenvolvimento de metástases no sistema nervoso central é um processo sequencial complexo e inter-relacionado denominado coletivamente de cascata de invasão-metástase (LAMBERT; PATTABIRAMAN; WEINBERG, 2017). Acredita-se que fatores como a agressividade das células tumorais disseminadas, a resistência aos tratamentos sistêmicos e a seleção clonal de células tumorais com tropismo cerebral especial contribuam para o desenvolvimento de metástases no SNC (LAAKMANN *et al.*, 2019).

A cascata de invasão-metástase é iniciada pela migração, invasão local de células na matriz extracelular circundante e intravasão na vasculatura. As células que sobrevivem às condições rigorosas existentes na corrente sanguínea (por exemplo a força de cerrilhamento), se aderem às células endoteliais cerebrais, extravasam para o parênquima cerebral através da BBB, adaptam-se ao novo microambiente para formar células dormentes ou micrometástases multicelulares e, eventualmente, prosperar no novo ambiente para estabelecer metástases macroscópicas clinicamente detectáveis (WANLEENUWAT; IWANOWSKI, 2020). Os depósitos metastáticos no cérebro geralmente se formam principalmente na junção da substância branca e cinzenta, com cerca de 80% dessas lesões envolvendo o cérebro. As lesões no cerebelo e no tronco cerebral, combinadas, representam os 20% restantes de todas as lesões (CARBONELL *et al.*, 2009; RAHMATHULLA; TOMS; WEIL, 2012).

A disseminação das células tumorais representa a etapa inicial da cascata metastática; nesta etapa as células adquirem características que as capacitam para deixar o sítio primário e migrar para tecidos distantes (LAMBERT; PATTABIRAMAN; WEINBERG, 2017). A disseminação metastática é um processo ineficiente, o que significa que apenas um pequeno subconjunto de células do tumor primário tem potencial para formar metástases (FIDLER, 2002; JOYCE; POLLARD, 2009). Em modelos animais experimentais, foi demonstrado que menos de 0,1% das células que deixam o tumor primário formam metástase (LUZZI *et al.*, 1998). Durante a disseminação metastática, as células de um tumor primário podem invadir os tecidos circundantes como células isoladas ou coletivamente, como um grupo (FARES *et al.*, 2020).

A cascata de invasão-metástase pode ser desencadeada por fatores genéticos e epigenéticos que resultam em instabilidade cromossômica causada por erros contínuos na segregação cromossômica durante a mitose (FARES *et al.*, 2020). As falhas na segregação

cromossômica além de alimentar a heterogeneidade tumoral, desencadeiam uma resposta autônoma ao DNA citosólico e mantém as células em um estado pró-metastático altamente inflamado (BAKHOUM *et al.*, 2018). Os sinais liberados em resposta à inflamação induzem o processo de transição epitelial mesenquimal (TEM). A TEM confere propriedades críticas para a invasão e disseminação metastática de células epiteliais. Essas propriedades incluem aumento da motilidade e capacidade de degradar componentes da matriz extracelular, invasão da membrana basal e de vasos sanguíneos (FARES *et al.*, 2020; LÓPEZ-NOVOA; NIETO, 2009).

Ao intravasarem, essas células persistem na corrente sanguínea como células tumorais circulantes (CTCs), sendo capazes de evadir a vigilância imune até a sua extravasão em órgãos distantes (WARD *et al.*, 2021). Enquanto na corrente sanguínea, a interação entre CTCs e plaquetas desempenha um papel crucial no processo metastático. É sabido que as plaquetas interagem com as CTCs, conferindo-lhes proteção contra diversos ataques do hospedeiro, como ataques imunológicos, apoptose e estresse de cisalhamento (ANVARI; OSEI; MAFTOON, 2021; LOU *et al.*, 2015; WARD *et al.*, 2021). Embora os mecanismos pelos quais as plaquetas agem sejam pouco compreendidos, há evidências de que as células tumorais primárias expressam mediadores agonísticos, como adenosina difosfato (ADP), trombina, thromboxane A2 (TXA2) e proteinases que aumentam a agregação plaquetária induzida por células tumorais (LOU *et al.*, 2015). Essas plaquetas, chamadas de “plaquetas ativadas” se ligam à superfície das CTCs por uma ponte GPIIb-IIIa-fibrinogênio e P-selectina, formando um "manto plaquetário" protetor que dificulta o reconhecimento dessas células pelo sistema imunológico, aumentando, assim, a taxa de sobrevivência das CTCs (LOU *et al.*, 2015). Esse revestimento protetor não apenas resguarda as CTCs contra ataques imunológicos, mas também reduz a exposição delas ao estresse de cisalhamento induzido pelo fluxo plasmático, contribuindo para a viabilidade dessas células. Além disso, foi descoberto que as plaquetas promovem a formação de nichos metastáticos precoces e auxiliam no recrutamento de outras células, como os granulócitos, independentemente dos sinais tumorais, através da liberação de quimiocinas específicas (WARD *et al.*, 2021).

Tanto a intravasão quanto a extravasão de células tumorais requerem a ruptura das junções endoteliais para que as células atravessem o endotélio — um processo conhecido como migração transendotelial mediado por metaloproteinase (REYMOND; D’ÁGUA; RIDLEY, 2013). A adesão das células neoplásicas ao endotélio cerebral e a subsequente promoção da parada circulatória são etapas críticas no processo de formação da MC. Esta interação envolve

a expressão de moléculas de adesão celular incluindo E-selectina, VCAM-1, ALCAM, ICAM-1, VLA-4 e α4 e a modulação da função das células endoteliais (SOTO *et al.*, 2014).

Uma vez aprisionadas, as células tumorais superexpressam enzimas associadas à mitogênese e fatores de crescimento, incluindo COX2 e HBEGF (que codifica um ligante para o receptor do fator de crescimento epidérmico, EGFR), permitindo a migração celular através da BBB. Curiosamente, as células tumorais também são capazes de aumentar a expressão da catepsina S, uma protease que é predominantemente expressa por leucócitos, para clivar as moléculas de adesão juncional que mantêm a integridade da BBB permitindo assim que as CTCs passem através da BBB (SCHULZ *et al.*, 2019).

A extravasão de células tumorais, a semeadura e a formação de micrometástases são mediados por uma combinação de proteínas circulantes, como fator de crescimento endotelial vascular (VEGF), metaloproteinases de matriz (MMPs), proteinase catepsina S, entre outras, que são produzidas por células tumorais ou células do microambiente tumoral (da sigla, em inglês, TME, *tumor microenvironment*) (ACHROL *et al.*, 2019). Uma vez localizadas no SNC, as células tumorais disseminadas (CTDs) encontram um microambiente fundamentalmente distinto do seu local de origem. Esse microambiente está em proximidade com células imunológicas e residentes cerebrais, como astrócitos, micróglia e neurônios. As CTDs têm a capacidade de entrar em um estado inativo ao ingressar no microambiente do SNC (ACHROL *et al.*, 2019; SAUER *et al.*, 2021). Enquanto a maioria das CTDs pode sofrer apoptose, aquelas que conseguem iniciar programas bem-sucedidos de adaptação e sobrevivência entram em um estado de dormência (ACHROL *et al.*, 2019; SAUER *et al.*, 2021). Algumas CTDs dormentes podem permanecer como células quiescentes solitárias ou formar pequenos aglomerados de células quiescentes. O microambiente do nicho metastático e sua remodelação desempenham um papel crucial em determinar o destino das CTDs latentes residentes, induzindo mecanismos intrínsecos nas células que resultam na saída do estado de dormência tumoral (ACHROL *et al.*, 2019; SAUER *et al.*, 2021).

A interação entre células tumorais e TME é crítica para o crescimento após a semeadura de células (MORAVAN *et al.*, 2020). Após extravasar, células tumorais individuais são imediatamente cercadas por astrócitos reativos que atuam como uma eficiente primeira linha de proteção no SNC, reduzindo o número de células com potencial de iniciar metástases. Essa defesa natural contribui, em parte, para a alta ineficiência da colonização do cérebro pelas células tumorais (VALIENTE *et al.*, 2018; WASILEWSKI *et al.*, 2017). No entanto, apesar

dessa defesa robusta, algumas células conseguem sobreviver e persistir no nicho perivascular, em proximidade com as células-tronco neurais. Nesse local, as células tumorais têm maior acesso a nutrientes e oxigênio, estabelecem contato com a lâmina basal dos capilares e desfrutam de um acesso preferencial aos fatores de crescimento angiócrinos produzidos pelas células endoteliais (VALIENTE *et al.*, 2018).

A proliferação de células que iniciam metástases pode resultar no estabelecimento de um número variável de micrometástases no cérebro. Algumas micrometástases podem interagir fisicamente com os astrócitos, que representam um componente crucial do microambiente cerebral (CAMPBELL *et al.*, 2022; NATHOO *et al.*, 2005). Os astrócitos, como componente principal do microambiente cerebral, desempenham um papel crucial na modulação do comportamento das células tumorais e na influência do microambiente imunológico. Quando entram em contato com células tumorais, os astrócitos exibem um fenótipo reativo, liberando uma grande quantidade de fatores de crescimento e participando de vias de sinalização que afetam a proliferação, invasão tumoral e o microambiente imunológico tumoral (ZHANG *et al.*, 2020). A influência direta dos astrócitos na invasão e metástase de células tumorais é evidente em estudos que demonstram sua acumulação em torno de focos metastáticos durante o processo de extravasão, frequentemente observados nas proximidades de tumores cerebrais metastáticos em modelos animais e pacientes humanos (WANG *et al.*, 2013).

Em resposta ao interferon tipo I, os astrócitos ativados pelas células cancerígenas metastáticas demonstraram facilitar a sobrevivência, o crescimento e a migração das células tumorais em diferentes estágios de crescimento metastático (MA *et al.*, 2023). Além disso, a caracterização de astrócitos reativos associados a tumores (da sigla em inglês, TARAs, *tumor-associated reactive astrocytes*) em gliomas revelou que essas células representam cerca de 30% das células no glioma, desempenhando papéis importantes no microambiente tumoral. Os TARAs foram identificados como um subtipo distinto, marcado pelo aumento da proliferação e ativação da via JAK/STAT, contribuindo para a complexa interação entre astrócitos e células tumorais em gliomas (ZHANG *et al.*, 2023).

1.4 Fatores genéticos associados a formação das metástases cerebrais no CPCNP

Apesar dos progressos nos estudos genômicos, transcritômicos e proteômicos, até o momento, não existem pesquisas que tenham elucidado completamente os mecanismos e os

genes/proteínas envolvidos na formação de MC em pacientes com câncer de pulmão. De fato, muitas das alterações genéticas que levam a essa complicação clínica ainda permanecem pouco compreendidas (POPPER, 2016).

Evidências crescentes sugerem que a metástase resulta da ativação inadequada de um programa genético denominado 'crescimento invasivo', um processo fisiológico que tipicamente ocorre durante o desenvolvimento embrionário e a regeneração pós-natal de órgãos. Esse processo é impulsionado pelo proto-oncogene *MET* (FARES *et al.*, 2020; LIU *et al.*, 2017; PATEL *et al.*, 2021). No caso de MC de câncer de pulmão, a ativação de *MET* e do receptor RON (Recepteur d'Origine Nantais), também conhecido como receptor-1 estimulador de macrófagos (MSTR1) – o qual pertence à mesma família de receptores de tirosina quinase do protoncogene *MET*, mostraram uma frequência aumentada de mutações e parecem ter um importante papel na ativação do crescimento invasivo (MILAN *et al.*, 2018; STELLA *et al.*, 2019). Mecanismos adicionais foram relatados envolvendo as vias de sinalização WNT/TCF, as quais atuam por meio de mediadores do fator de transcrição HOXB9 (Homeobox B9) e Lymphoid Enhancer Binding Factor 1 (LEF1), com papel no estímulo à invasão e proliferação de células tumorais (NGUYEN *et al.*, 2009).

Mutações no proto-oncogene GTPase KRAS (*KRAS*) e quinase hepática B1 (*LKB1*) foram consideradas um fator de risco para MC em CPCNP (ZHAO *et al.*, 2014). Pacientes com mutações ativadoras no domínio da quinase do *EGFR* ou com rearranjos da quinase do linfoma anaplásico (*ALK*) tiveram um risco maior de desenvolver MC (CHUN *et al.*, 2012; LI *et al.*, 2017). Em pacientes com mutações em *EGFR*, a deleção do exon 19 exibiu uma tendência potencial para promover MC em comparação com a mutação do exon 21 (LI *et al.*, 2017). Sugere-se que o risco aumentado de MC em pacientes com CPCNP com mutações ativadoras em *EGFR* e *ALK*, esteja associado ao uso de inibidores da tirosina quinase (TKIs), o que permite prolongar a sobrevida global e, consequentemente, proporcionar maiores chances de desenvolver MC durante o curso da doença (LI *et al.*, 2017; SINGH *et al.*, 2020).

Alterações genômicas em 182 genes relacionados ao câncer e 37 íntrons de 14 genes frequentemente rearranjados no câncer foram avaliadas por sequenciamento de nova geração (da sigla em inglês, NGS, *Next Generation Sequencing*) em pares de tumores metastáticos primários e seus respectivos pareados, compreendendo 15 pacientes (VIGNOT *et al.*, 2013). Foram observadas 63 alterações recorrentes conhecidas, sendo 32 no tumor primário e 31 na metástase. Conforme antecipado, as mutações na proteína tumoral P53 (TP53) foram as mais

frequentes. Além disso, uma taxa de concordância de 94% de alterações recorrentes entre tumor primário e metástases correspondentes foi observada, sugerindo que o tumor primário pode ser útil para identificar, pelo menos parcialmente, alterações somáticas recorrentes nas metástases (VIGNOT *et al.*, 2013).

Em pacientes com adenocarcinoma de pulmão, um estudo do tumor primário de sete pacientes com MC identificou que os níveis de expressão proteica de cinco genes (*TYMS*, *CDK1*, *HJURP*, *CEP55* e *KIF11*) foi preditivo de metástase cerebral nestes pacientes (FU *et al.*, 2020). Esse mesmo estudo mostrou que as vias de sinalização preferencialmente relacionadas à MC foram as vias de reparo a danos ao DNA, em vez da via de transição epitelial-mesenquimal, que deve ser significativa para invasão tumoral e metástase (FU *et al.*, 2020). Outro estudo avaliou um painel seleto de 160 genes associados ao câncer, por NGS, em CPCNP com MC sincrônicas, metacrônicas e extracranianas de 39 amostras e identificou mutações em genes envolvidos na sinalização PI3K/AKT nos tumores primários e nas MC (WILSON *et al.*, 2018). Variantes únicas foram detectadas em 20 genes (*TP53, SMAD4*, *SF3B1*, *NOTCH2*, *MTOR*, *MSH6*, *KRAS*, *KMT2D*, *KDM6A*, *IKZF1*, *GNAS*, *FANCD2*, *ERCC5*, *EP300*, *CREBBP*, *CDK12*, *BRCA2*, *BCL6*, *ATM*, *ABL1*) em mais 33% dos pacientes com MC. Este painel de genes foi sugerido para identificar pacientes com risco de desenvolver MC (WILSON *et al.*, 2018).

Através do sequenciamento direcionado de 416 genes pertinentes ao câncer em amostras de tumor primário de CPCNP e metástases cerebrais (MC) pareadas de 61 pacientes, foram identificadas alterações nas vias de sinalização CDK4/CCND1, CDKN2A/2B e PI3K nas MC, bem como mutações nos genes *EGFR*, *KRAS*, *TP53* e *ALK*, concordantes entre os tumores primários e as MC. Contudo, observou-se que as MC apresentaram mais mutações específicas da lesão do que o tumor primário, sugerindo uma evolução genômica nas MC, com a ativação exclusiva de mecanismos oncogênicos nessas lesões (WANG *et al.*, 2019).

Esses dados indicam que as células tumorais metastáticas que conseguiram colonizar o tecido cerebral desenvolvem rotas alternativas às seguidas no tumor primário, principalmente devido à natureza hostil do novo microambiente. Tais informações têm implicações significativas na concepção de abordagens terapêuticas específicas para MC, além de serem relevantes para o desenvolvimento de biomarcadores que possam determinar com maior precisão quais pacientes estão em risco de desenvolver metástases no cérebro.

1.5 Aplicações das tecnologias de análise transcriptômica no estudo do câncer

Atualmente, as tecnologias de sequenciamento de nova geração (NGS) proporcionam a oportunidade de gerar vastas e crescentes quantidades de dados de maneira fácil, econômica, abrangente e com alta qualidade. O sequenciamento de RNA (RNA-seq), desenvolvido há mais de uma década, tornou-se uma ferramenta onipresente na biologia molecular, desempenhando um papel fundamental na compreensão da função genômica (STARK; GRZELAK; HADFIELD, 2019).

O RNA-seq apresenta diversas vantagens em relação a outras tecnologias NGS. Por exemplo, tanto o sequenciamento completo do exoma (WES, da sigla em inglês, *whole exome sequencing*) quanto o RNA-seq são capazes de detectar mutações em regiões codificadoras (OZSOLAK; MILOS, 2011). No entanto, o RNA-seq não requer etapas de enriquecimento de éxon, resultando em um custo-benefício mais favorável quando comparado ao WES (CHEPELEV *et al.*, 2009; KU *et al.*, 2012). Na detecção de variantes não codificadoras patogênicas, o RNA-seq pode concentrar-se em um pequeno grupo de genes alterados e seus elementos reguladores, o que é mais vantajoso do que estudos baseados em dados de sequenciamento de DNA (BRONSTEIN *et al.*, 2020; YÉPEZ *et al.*, 2022). Além disso, a tecnologia de RNA-seq permite a quantificação da abundância de transcritos, identificação de eventos de splicing alternativo, detecção de transcrição de fusão, detecção de polimorfismo de nucleotídeo único (SNPs), descoberta de novas regiões transcritas ainda não caracterizadas, e possibilita a identificação de transcritos raros ou codificadores de proteínas de baixa abundância, bem como éxons ainda não anotados e mutações em genes associados ao câncer (KU *et al.*, 2012). Esses fatores tornam o RNA-seq uma ferramenta útil para o estudo da regulação da expressão gênica eucariótica, dos mecanismos envolvidos na iniciação e progressão do câncer, das estratégias de tratamento, e proporcionam perspectivas promissoras na compreensão de doenças genéticas (STENSON *et al.*, 2017).

Diversos estudos demonstraram a aplicação bem-sucedida de RNA-seq na descoberta de novas mutações condutoras no câncer (BAILEY *et al.*, 2018; CAO *et al.*, 2020; LI *et al.*, 2019; SHI *et al.*, 2020) e suas aplicações na oncologia clínica (HEYER *et al.*, 2019; WANG *et al.*, 2020). Até o momento, a maioria dos estudos moleculares de MC no câncer de pulmão, utilizam como estratégia o sequenciamento completo do exoma (BRASTIANOS *et al.*, 2015; PAIK *et al.*, 2015; SHIH *et al.*, 2020), microarranjos (DAI *et al.*, 2020; GACHECHILADZE

et al., 2013; GRINBERG-RASHI *et al.*, 2009; ZHAO *et al.*, 2014) e sequenciamento de DNA visando genes associados ao câncer (MACHADO-RUGOLO *et al.*, 2019; PREUSSER *et al.*, 2015; VIGNOT *et al.*, 2013; WANG *et al.*, 2019; WILSON *et al.*, 2018), investigando alterações moleculares em tumores primários para prever o desenvolvimento de metástases. ***No entanto, foi demonstrada uma vasta diversidade genética entre tumores primários e MC em diversos tipos de tumores primários*** (ACHROL *et al.*, 2019)***. A avaliação da heterogeneidade intra-lesão, através da amostragem de várias regiões de MC únicas, bem como da heterogeneidade inter-lesão, por meio da amostragem de várias MC anatomicamente e temporalmente distintas no mesmo paciente, evidenciou que as MC compartilham mutações que não são detectadas no tumor primário. Além disso, observou-se que há mais alterações moleculares comuns entre as MC do que entre estas e o tumor primário*** (BRASTIANOS *et al.*, 2015).

Além do RNA-seq, o sequenciamento de RNA de célula única (scRNA-Seq, do inglês, *single-cell RNA sequencing*) tem emergido como uma poderosa ferramenta na pesquisa do câncer, fornecendo *insights* de alta resolução sobre o cenário celular e molecular dos tumores (PURAM *et al.*, 2017). Recentemente, o scRNA-Seq foi empregado para explorar o TME em MC de câncer de mama e pulmão. Song et al. perfizeram o perfilamento de 10.896 células, identificando subpopulações intratumorais variadas (SONG *et al.*, 2023). Entretanto, as informações histológicas específicas para as amostras de câncer de pulmão foram limitadas. Em um estudo prévio, Sun *et al*. analisaram 61.867 células de gliomas e MC de adenocarcinoma de pulmão, destacando subpopulações celulares heterogêneas. Neste estudo, evidenciou-se que células de câncer de pulmão reprogramaram o TME para um estado imunossuprimido, envolvendo micróglia, macrófagos, células endoteliais e células T CD8+. Adicionalmente, um subconjunto de macrófagos foi associado a um mau prognóstico (SUN *et al.*, 2022). Outros estudos também exploraram os perfis transcriptômicos unicelulares em MC de câncer de pulmão (KIM *et al.*, 2020; WANG *et al.*, 2023).

Embora as tecnologias NGS sejam amplamente utilizadas na pesquisa atual sobre o câncer, é crucial reconhecer a significativa contribuição das técnicas baseadas em microarranjos (*microarray* em inglês) ao longo do tempo. As metodologias de microarranjos desempenharam um papel fundamental no avanço do conhecimento no campo do câncer, oferecendo abordagens valiosas para a análise da expressão gênica (AHN *et al.*, 2014), identificação de biomarcadores (JIN *et al.*, 2020), compreensão da heterogeneidade tumoral (BARRY *et al.*, 2010) e modelagem preditiva (LAI *et al.*, 2020).

Em resumo, as vantagens derivadas do uso de dados transcriptômicos destacam-se pela abundância de informações fornecidas. Essas abordagens possibilitam uma exploração mais minuciosa e abrangente dos aspectos genéticos, moleculares e celulares relacionados à formação de MC no câncer de pulmão. Essas tecnologias, em última análise, representam ferramentas valiosas para identificar os principais condutores das MC no câncer de pulmão, apontar alvos terapêuticos e descobrir biomarcadores associados a esse processo altamente complexo.

Os biomarcadores identificados por meio dessa análise podem resultar em aplicações clínicas relevantes, contribuindo para o desenvolvimento de estratégias terapêuticas mais precisas e beneficiando os pacientes afetados por essa doença letal.

1.6 Integração de conjuntos de dados transcriptômicos

O progresso das tecnologias de sequenciamento de nova geração representa uma oportunidade única para produzir dados de alta qualidade em larga escala (SATAM *et al.*, 2023)**.** Essa tecnologia tem sido fundamental para o estabelecimento e a disseminação generalizada de repositórios públicos nos quais os dados provenientes das mais diversas tecnologias de sequenciamento incluindo genômica, transcriptômica, epigenômica, metabolômica, proteômica entre outros podem ser depositados na forma bruta ou processados e estão disponíveis para acesso da comunidade científica. Essa prática, além de propiciar a reprodutibilidade e promover a transparência na pesquisa, oferece a oportunidade de preservação de informações para a reutilização dos dados em análises subsequentes e futuras investigações (SIELEMANN; HAFNER; PUCKER, 2020).

Repositórios amplamente reconhecidos, como o Omnibus de Expressão Gênica (da sigla, em inglês, GEO, *Gene Expression Omnibus*) (BARRETT *et al.*, 2013; EDGAR; DOMRACHEV; LASH, 2002), o Banco de Dados de Genótipos e Fenótipos (da sigla, em inglês, dbGaP, *The database of Genotypes and Phenotypes*) (MAILMAN *et al.*, 2007) e o Atlas do Genoma do Câncer (da sigla, em inglês, TCGA, *The Cancer Genome Atlas*) (CANCER GENOME ATLAS RESEARCH NETWORK *et al.*, 2013), são fontes de valor inestimável para a pesquisa em larga escala. O TCGA, por exemplo, fornece um recurso rico para o estudo do câncer, com mais de 20.000 amostras abrangendo 33 tipos de câncer, oferecendo mais de 2,5 petabytes de dados ômicos representados por dados genômicos, transcriptômicos,

epigenômicos e proteômicos. Esses recursos têm possibilitado melhorar a compreensão da base molecular do câncer e aumentado as possibilidades de diagnóstico, tratamento e prevenção do câncer. No caso do adenocarcinoma pulmonar, por exemplo, dados do TCGA permitiram a descoberta de mais evidências sobre a alta taxa de mutações neste tipo de câncer, a ativação de várias vias receptoras de tirosina quinase e a categorização do câncer em três subtipos distintos com base nos dados de expressão gênica (CANCER GENOME ATLAS RESEARCH NETWORK, 2014).

Além do TCGA, o GEO destaca-se como um recurso de dados de acesso aberto, impulsionado por conjuntos de dados carregados por diversos usuários. Até dezembro de 2023, o GEO abrigava uma impressionante coleção de 4.348 conjuntos de dados, originados de mais de 100 organismos distintos. Este vasto repositório de dados oferece acessibilidade e exploração eficazes por meio de ferramentas baseadas na web, intuitivas e de fácil utilização. Embora o GEO hospede diversos tipos de dados, abrangendo linhagens celulares e amostras não tumorais, entre os tipos de tumores sólidos, o câncer de mama e o câncer cerebral (incluindo glioma, meduloblastoma e glioblastoma) são as categorias predominantes (DAS *et al.*, 2020).

Com uma riqueza de conjuntos de dados transcriptômicos, o GEO emergiu como uma plataforma abrangente, fornecendo dados extensos e anotados de perfis de expressão gênica em tecidos tumorais. Mais notavelmente, essa plataforma oferece a oportunidade de integrar esses conjuntos de dados transcriptômicos com outras camadas ômicas. Apesar das vantagens, é crucial notar que no GEO, a limitação de recursos para integrar diferentes conjuntos de dados de expressão de vários estudos representa um desafio. Essa limitação destaca a necessidade contínua de desenvolvimento e implementação de estratégias mais eficazes para facilitar a integração e análise conjunta de dados provenientes de diversas fontes.

Estudos recentes concentraram-se na integração de diferentes conjuntos de dados ômicos (por exemplo, a combinação de dados genômicos e transcriptômicos) para abordar questões biológicas complexas (FRAUNHOFFER *et al.*, 2022; KHADIRNAIKAR; SHUKLA; PRASANNA, 2023; RUAN *et al.*, 2022). As metodologias de integração de dados visam extrair conhecimento biológico adicional de múltiplos conjuntos de dados que não podem ser obtidos apenas a partir de conjuntos de dados individuais, por exemplo, essas metodologias podem lidar com vários conjuntos de dados que diferem em poder estatístico, tipo e origem (HEO *et al.*, 2021). No entanto, embora cada uma das tecnologias ômicas ofereça uma visão

do sistema complexo, esses eventos são bastante interdependentes (ou interativos). Assim, ao combinar vários dados ômicos diferentes para descobrir as assinaturas biológicas, é um desafio incorporar diferentes camadas biológicas de informação para prever resultados fenotípicos (tumor/normal, estágio inicial/tardio, sobrevivência, etc.) (HUANG; CHAUDHARY; GARMIRE, 2017). Na última década, vários métodos foram propostos para abordar as diferentes facetas do problema de integração de dados, incluindo inovações em aprendizagem automática (CAI *et al.*, 2022; GLIGORIJEVIĆ; PRŽULJ, 2015; LAPATAS *et al.*, 2015).

Além da abordagem da multiômica integrada, uma estratégia adicional consiste na integração de diversos conjuntos de dados oriundos da mesma tecnologia, como é o caso da integração de múltiplos conjuntos de RNA-Seq ou scRNA-seq, por exemplo (PANG *et al.*, 2022; PRAZANOWSKA; LIM, 2023). O número de conjuntos de dados scRNA-seq disponíveis publicamente contendo amostras de vários tecidos e espécies aumentou muito na última década, com o GEO sendo uma das plataformas mais populares dedicadas à deposição de tais dados. No entanto, o pequeno tamanho da coorte, a inclusão de tipos de células limitados e a anotação insuficiente das populações de células são obstáculos comuns à reutilização eficiente dos dados. Portanto, diversas estratégias foram desenvolvidas para integração dos dados do scRNA-seq e correção de diferenças técnicas entre as amostras, também denominadas como efeito de lote. Dentre essas estratégias, Harmony (KORSUNSKY *et al.*, 2019) e Seurat (HAO *et al.*, 2023) são comumente recomendadas. A integração de conjuntos de dados scRNA-seq é particularmente valiosa, pois proporciona uma análise abrangente de características celulares em diversas condições. Essa abordagem oferece maior poder estatístico para detectar fenômenos raros, maior precisão na identificação do tipo de célula e *insights* biológicos aprimorados por meio de correlações entre conjuntos de dados.

Neste trabalho, realizamos a integração de dados transcriptômicos (RNA-Seq, microarray e scRNA-Seq) disponíveis em bancos de dados públicos, empregando métodos avançados de bioinformática. Nosso objetivo principal é elucidar os condutores transcritômicos das metástases cerebrais do adenocarcinoma de pulmão, um dos subtipos mais incidentes e letais desta doença. A identificação de biomarcadores tem potencial aplicação no desenvolvimento de terapêuticas moleculares; desta forma, estudos como este têm o poder de impactar significativamente o tratamento e, consequentemente, a sobrevida dos pacientes.

2. Hipótese

A hipótese deste estudo é que a integração de múltiplos conjuntos de dados de transcriptoma global e de células únicas é capaz de identificar alterações moleculares e celulares específicas da metástase cerebral de adenocarcinoma de pulmão.

3. Objetivos

3.1 Objetivo Geral

Caracterizar o transcritoma codificador da metástase cerebral de adenocarcinoma de pulmão a partir da integração de dados de sequenciamento de RNA (RNA-Seq) e sequenciamento de RNA de célula única (scRNA-seq), identificando perfis imunológicos, dinâmica celular e características moleculares associadas à doença.

3.2 Objetivos Específicos Capítulo 1

a. Realizar uma revisão abrangente da literatura para resumir o estado atual do conhecimento sobre os mecanismos de disseminação metastática do câncer de pulmão para o cérebro;
b. Apresentar uma visão abrangente das terapias existentes para pacientes diagnosticados com metástases cerebrais de câncer de pulmão.

3.3 Objetivos Específicos Capítulo 2

a. Comparar o perfil transcritômico de metástases cerebrais com tumor primário;
b. Anotar funcionalmente e enriquecer vias dos transcritos com expressão alterada nas metástases cerebrais;
c. Identificar o imunofenótipo das metástases cerebrais;
d. Caracterizar *(in silico)* a composição celular da metástase cerebral de adenocarcinoma de pulmão.

3.4 Objetivos Específicos Capítulo 3

a) Analisar *(in silico)* o microambiente tumoral de metástases cerebrais e tumores primários de adenocarcinoma de pulmão;
b) Explorar as interações moleculares entre as células imunes e não-imunes que compõe o microambiente tumoral;
c) Investigar como o microambiente tumoral influencia a progressão da metástase cerebral.

4. Materiais, métodos, resultados e discussão: introdução aos capítulos

Os materiais, métodos, resultados e discussão estão organizados em capítulos apresentados no formato de artigos científicos.

O capítulo 1, intitulado "**Advances in the Molecular Landscape of Lung Cancer Brain Metastasis**", apresenta uma visão abrangente da literatura atual sobre as principais fases da disseminação metastática do câncer de pulmão para o cérebro. Explora a influência do microambiente tumoral e os determinantes moleculares que impulsionam a progressão desse processo. Além disso, destaca os progressos significativos alcançados no diagnóstico molecular da metástase cerebral por meio da biópsia líquida, enquanto também discute perspectivas inovadoras de estratégias de tratamento para as metástases cerebrais. Esse capítulo foi publicado na revista *Cancers* (PMID: 36765679).

O capítulo 2, intitulado "**Identifying New Contributors to Brain Metastasis in Lung Adenocarcinoma: A Transcriptomic Meta-Analysis**", detalha os resultados de uma abrangente meta-análise transcriptômica. Essa análise incorpora dados de RNA-Seq total (também chamado de *bulk* RNA-Seq), microarrays e scRNA-Seq, identificando os principais impulsionadores da metástase cerebral de adenocarcinoma de pulmão. Os achados deste estudo contribuem para a compreensão do panorama celular e molecular envolvido na formação das metástases cerebrais associadas ao adenocarcinoma de pulmão. Esse capítulo também foi publicado na revista *Cancers* (PMID: 37760494).

Por fim, o capítulo 3, intitulado "**Comprehensive Analysis of Lung Adenocarcinoma and Brain Metastasis through Integrated Single-Cell Transcriptomics**", detalha o microambiente tumoral das metástases cerebrais e dos tumores primários (estadiamentos I, II, III e IV) de adenocarcinoma de pulmão, revelando a composição celular, incluindo células imunes e não imunes, presentes no microambiente tumoral. Além disso, são investigadas as interações celulares e as características moleculares associadas à formação da metástase no cérebro. Os achados deste estudo esclarecem os aspectos moleculares da metástase cerebral de adenocarcinoma de pulmão, fornecendo conhecimento essencial para pesquisas futuras e enfatizando o potencial das imunoterapias e abordagens personalizadas. Este capítulo foi submetido à revista *International Journal of Molecular Sciences* (IJMS).

Os materiais suplementares e as referências bibliográficas citadas ao longo dos capítulos serão devidamente listados ao final da tese, seguindo as normativas específicas de cada revista. Essas informações serão apresentadas em seções intituladas "Materiais Suplementares" e "Referências Bibliográficas", respectivamente.

4.1 Capítulo 1

Advances in the Molecular Landscape of Lung Cancer Brain Metastasis

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Simple Summary

Patients with lung cancer have high rates of brain metastasis (BM). Despite available therapies, patient prognosis is poor. Studies have shown genetic alterations associated with the metastatic spread of lung cancer cells. However, the precise mechanisms governing BM are still unclear. In this review, we comprehensively describe the major steps of metastatic spread of lung cancer to the brain, addressing the influence of the tumor microenvironment and the molecular determinants of progression. Furthermore, we highlight the advances in the molecular diagnostics of BM by liquid biopsies and discuss novel treatment strategies.

Abstract

Lung cancer is one of the most frequent tumors that metastasize to the brain. Brain metastasis (BM) is common in advanced cases, being the major cause of patient morbidity and mortality.

BMs are thought to arise via the seeding of circulating tumor cells into the brain microvasculature. In brain tissue, the interaction with immune cells promotes a microenvironment favorable to the growth of cancer cells. Despite multimodal treatments and advances in systemic therapies, lung cancer patients still have poor prognoses. Therefore, there is an urgent need to identify the molecular drivers of BM and clinically applicable biomarkers in order to improve disease outcomes and patient survival. The goal of this review is to summarize the current state of knowledge on the mechanisms of the metastatic spread of lung cancer to the brain and how the metastatic spread is influenced by the brain microenvironment, and to elucidate the molecular determinants of brain metastasis regarding the role of genomic and transcriptomic changes, including coding and non-coding RNAs. We also present an overview of the current therapeutics and novel treatment strategies for patients diagnosed with BM from NSCLC.

Keywords: brain metastasis; lung cancer; microenvironment; molecular mechanisms; coding and non-coding RNAs; therapeutic strategies

1. Introduction

Non-Small Cell Lung Cancer (NSCLC) accounts for the majority (85%) of lung cancer cases, with adenocarcinoma and squamous cell carcinoma being the most common histological subtypes [1]. Tobacco smoking, air pollution, and exposure to radiation and occupational carcinogens are among the most common risk factors [2]. Increased incidence of lung cancer has been observed in never-smokers and younger individuals and is frequently associated with the adenocarcinoma subtype. It is expected that by 2040, the worldwide incidence of lung cancer will increase from 2 to over 3 million cases per year, and the number of annual deaths will rise from 1.8 to over 2.9 million [3]. A limited proportion of lung cancer patients undergo surgery as the primary treatment since most patients (~75%) present locally advanced or distant metastatic disease at diagnosis and are not eligible for curative surgical treatment. Since current treatment strategies are focused on treating late-stage disease, patient prognosis remains dismal, with high mortality rates.

Metastatic disease is one of the main causes of patient death. Therefore, uncovering the mechanisms underlying metastasis is pivotal for improving therapeutic strategies and patient survival [4].

Previous treatment of advanced lung cancer was limited to cytotoxic chemotherapy, but the identification of oncogenic driver mutations in NSCLC has dramatically changed the therapeutic approaches in the past decades. Targeted therapy and immunotherapy have considerably improved survival in selected patients [1]. Large-scale genomic studies have enabled the identification of activating driver mutations associated with primary lung cancer. These findings contributed to advances in therapeutics with the development of tyrosine kinase inhibitors (TKIs), which led to improvements in patient survival. However, these therapies benefit a fraction of patients with lung adenocarcinoma harboring driver mutations. Mutations in genes such as the Epidermal Growth Factor Receptor (*EGFR*), Anaplastic Lymphoma Kinase (*ALK*), ROS1 Proto-Oncogene Tyrosine Kinase Receptor (*ROS1*), and Serine/Threonine-Protein Kinase BRAF (*BRAF*) are therapeutic targets in lung adenocarcinoma, and novel mutations may be introduced as targeted therapies [5]. Other activating mutations occur in oncogenes such as *KRAS* and are associated with worse prognosis, with no approved drugs able to efficiently inhibit *KRAS* activation [5,6,7] until the recent development of novel inhibitors such as sotorasib, shown to efficiently target the KRASp.G12C mutation in advanced solid tumors [8]. Another KRAS inhibitor, adagrasib, is under investigation to treat patients with progressive metastatic lung cancer [9]. In a phase 2 clinical trial, sotorasib, which specifically and irreversibly inhibits the KRASp.G12C mutation, was tested in a cohort of 126 NSCLC patients, with the majority having previously received systemic platinum-based chemotherapy combined with immunotherapy based on PD-1 or PD-L1 immune checkpoint inhibitors (ICIs). Results showed a complete response in 4/126 patients (3.2%) and a partial response in 42/126 patients (33.9%) with a median duration of response of 11.1 months. These data showed a clinical response for relapsed advanced *KRAS*-mutated NSCLC with disease control obtained in >80% of patients. Median progression-free survival and overall survival were 6.8 months and 12.5 months, respectively [10]. Novel treatments targeting *KRAS*-mutated tumors are promising; however, patient prognosis remains poor with modest progression-free and overall survival rates, likely due to disease heterogeneity. Recent reviews on targeted therapies with KRAS inhibitors including a combination with immunotherapies are available in [11,12,13].

Mutations in *EGFR* occur in 15–40% of adenocarcinoma cases, occurring more frequently in women of Asian ancestry and never-smokers. TKIs targeting *EGFR* mutations include the first-generation drugs gefitinib and erlotinib, and afatinib and osimertinib, second and third generation, respectively [5]. Although TKIs improved progression-free survival, mechanisms of acquired resistance are common and lead to disease progression in most patients [5]. *ALK* rearrangements are found in ~7% of patients with lung adenocarcinoma and are mutually exclusive with *KRAS* and *EGFR* mutations [6]. The identification of the *ALK* rearrangement is predictive for therapeutic targeting by crizotinib. New generations of ALK TKIs ceritinib, alectinib, and brigatinib have been used [5]. *ROS1* rearrangements occur in 1–2% of cases and are most common in adenocarcinoma patients, younger patients (<40 years old), females, and never-smokers. Crizotinib is used for patients with ROS1-positive tumors [6]. Somatic mutations in *BRAF* occur in ~3–8% of adenocarcinoma cases; of these, ~50% are *BRAF* V600E, which is predictive for vemurafenib and dabrafenib-based therapies [5]. Genomic studies have revealed additional driver mutations in NSCLC, which can be explored for targeted therapy.

Conversely, patients with squamous cell lung carcinoma, which represents about 20–30% of NSCLC, have limited treatment options. Treatment with biomarker-driven therapies targeting FGFR, PI3K, MET, EGFR, among others, failed to demonstrate activity in the Lung Cancer Master Protocol (Lung-MAP SWOG S1400). However, an ongoing phase 2 open label clinical trial (RAGNAR) showed evidence of efficacy for erdafitinib, a selective pan-FGFR tyrosine kinase inhibitor, in heavily pretreated patients with different *FGFR*-positive solid tumors, including squamous and non-squamous cell lung cancer [14].

In the past decade, immunotherapy based on immune checkpoint inhibitors (ICIs) has shown significant survival benefits for patients with advanced NSCLC. Cancer cells develop immune evasion mechanisms playing a pivotal role in cancer progression. Monoclonal antibodies, such as pembrolizumab and nivolumab, are directed to block the PD-1 receptor in T lymphocytes, preventing immune response inhibition [1,15]. Patients with advanced NSCLC treated with ICIs have improved survival in comparison to standard chemotherapy in both first- and second-line therapies. The efficacy of nivolumab monotherapy in pretreated advanced non-squamous and squamous cell lung cancer showed a 17% objective rate response (ORR) and a median of 17.0 months of response duration among patients [16]. The combination of different ICIs with distinct and complementary mechanisms to improve anti-tumor immunity, such as nivolumab targeting PD1 and ipilimumab targeting CTLA4 in T lymphocytes, was tested in a

phase 1, multi-cohort study showing high response rate and durable response with tolerable safety in NSCLC [17]. However, despite durable responses, not all patients benefit from ICI treatment [15], highlighting the importance of identifying biomarkers of immunotherapy response.

In this review, we describe the current state of knowledge regarding the molecular and cellular mechanisms involved in metastatic spreading of lung cancer cells to the brain. We discuss the influence of the brain microenvironment, including immune cells to support tumor cell growth. Moreover, a comprehensive discussion of genomic and transcriptomic alterations, including coding and non-coding RNAs, as genetic determinants of brain metastasis in NSCLC is presented. We also provide an overview of the current therapeutics, new treatment opportunities, and future directions for patients diagnosed with BM from NSCLC.

2. NSCLC Brain Metastasis

NSCLC frequently metastasizes to bone, brain, lung, and liver. BM accounts for most of the central nervous system (CNS) tumors, being observed in up to 40% of patients with different cancer types. Strikingly, BM is about 10 times more common than primary tumors affecting the CNS [18,19]. Patients with lung cancer have the highest rates of identified BMs [20]. Approximately 10–20% of NSCLC patients have BM at the time of diagnosis and approximately 40% will develop BM during the course of disease [21,22]. BMs often appear as multiple lesions, although one-third of patients present single lesions [23]. BM is highly prevalent in lung adenocarcinoma, markedly worsening patient outcomes, with a median survival of up to 15 months for treated patients [24].

The incidence of BM is probably underestimated since routine brain magnetic resonance image (MRI) screening in patients who do not present neurological symptoms is not recommended. Routine brain MRI would increase the detection of asymptomatic brain metastasis. However, its use as a populational guideline is controversial due to the high burden on the patients and the health care system [25,26]. In addition, a proportion of patients with negative screens may develop brain metastasis within one year [27]. Therefore, current guidelines support routine neuroimaging scans for more advanced clinical stages. Moreover, many studies frequently report the detection of BM at the time of initial diagnosis, but no

information is provided on the subsequent sites of metastatic involvement during the disease course [23]. BM is often associated with severe neurologic and cognitive difficulties that are responsible for patient morbidity and significantly decreased quality of life. Headache, followed by neurologic dysfunction, seizures, stroke-like symptoms, and/or subtle cognitive dysfunction are the most common symptoms [19]. In fact, BM is often detected based on neurological symptoms without a diagnosis of a primary lung tumor. Studies have reported that most BM originating from lung cancer is located in the supratentorial area of the brain [28] and its distribution depends on the mutational status of *EGFR* [29].

Leptomeningeal metastases are a subset of BMs that grow in the lining of the brain or spine and/or cerebrospinal fluid (CSF) and occur with or without brain parenchyma metastases. Leptomeningeal metastasis is less common, occurring in 3–5% of patients with advanced NSCLC, and has been recently reviewed elsewhere [30]. Its incidence has increased in subgroups of patients who have received targeted molecular therapy due to the extended survival time. The prognosis of patients with leptomeningeal metastasis from NSCLC is poor; however, it has improved from a median historical survival (pre-approval of contemporary systemic treatment) of 1–3 months to 3–11 months with the use of new therapies [30,31].

The prognosis of patients with BM depends on different factors such as primary tumor site and other prognostic indicators, including driver mutations. In lung adenocarcinoma, BM occurs in ~20–40% of patients with *ALK* rearrangements and ~25% of patients with *EGFR*-mutated tumors [32,33,34]. The graded prognostic assessment (GPA) is a prognostic index that helps estimate patient survival in the presence of BM. In addition, patient age, Karnofsky performance status (KPS), extracranial metastases, and number of BMs are diagnostic-specific prognostic indices for patients with NSCLC. GPA scores range from 0-4, from worst to best prognosis, and define survival times ranging from 3.0–14.8 months for NSCLC patients [35]. An update of the GPA prognostic index including molecular markers, the Lung-molGPA, added *EGFR* and *ALK* mutation status for patients with lung adenocarcinoma. Median patient survival ranged from 3.0–46.8 months, although only 4% of the patients showed the highest scores (3.5–4.0) with a median survival of ~46 months [36]. Extensive efforts have focused on predicting outcomes for patients who develop BMs.

3. The Development of Brain Metastasis Is a Complex, Multistage Process

The major steps of metastatic spread to the brain are the dissociation of cells from the primary tumor, invasion of surrounding stroma and basement membrane, cancer cell intravasation, extravasation, and breaking down of the blood–brain barrier (BBB) followed by CNS invasion and colonization [18]. BM arises through seeding of circulating tumor cells (CTCs) into the brain microvasculature. Tumor cells interact with the brain endothelium, increasing the adhesion of tumor cells and promoting circulatory arrest. Once trapped, tumor cells start the process of crossing the BBB, which is a crucial step in tumor dissemination to the brain. The BBB harbors tight and adherens junctions between the brain endothelial cells, which regulate the flow of ions and nutrients, establishing a selective permeability barrier that protects the brain from blood-derived toxins and restricts the migration of leukocytes and monocytes [37,38,39]. BBB permeability is highly increased during BM in lung cancer [40], allowing CTCs to penetrate the brain and promote BM development. Several mechanisms associated with BBB remodeling that facilitate the migration of tumor cells through the BBB have been identified, including the secretion of various proteases to degrade tight junction components [41,42,43]. For example, cancer cells overexpress enzymes associated with mitogenesis and growth factors, including prostaglandin-endoperoxide synthase 2 (COX2) and heparin-binding EGF-like growth factor (HBEGF), allowing cell migration through the BBB [44,45].

Interestingly, tumor cells are also able to increase the expression of cathepsin S, a protease that is predominantly expressed by leukocytes, to cleave the junctional adhesion molecules that maintain BBB integrity and thus help tumor cells to break down the BBB [46]. In addition, extravasation of tumor cells, seeding, and formation of micrometastases are mediated by a combination of circulating proteins, including vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), among others, which are produced by tumor cells or cells in the tumor microenvironment (TME). Therefore, metastatic formation is mediated by a combination of circulating molecules, mainly proteins secreted by tumor cells and cells in the TME [23,47] (**Figure 1**).

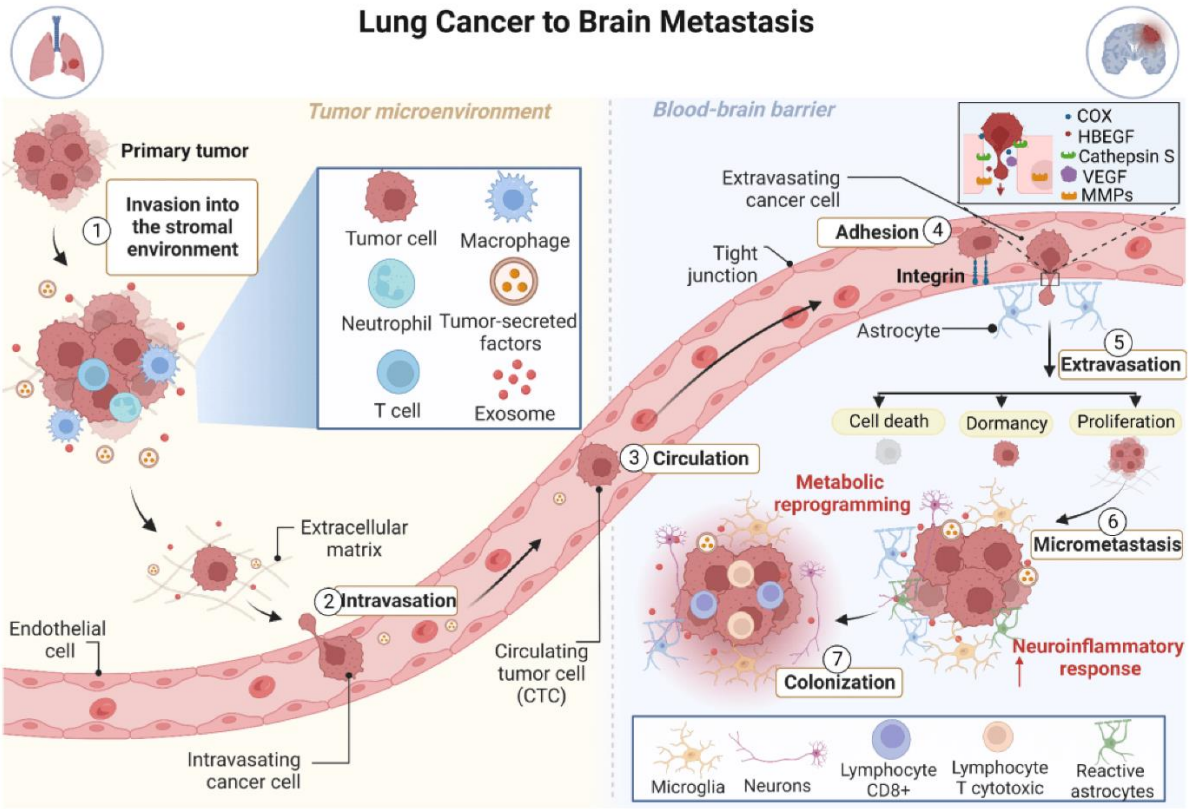

Figure 1. Schematic illustration of brain metastasis development through hematogenous dissemination. Initially, tumor cells at the primary site acquire invasive properties, break away from the primary tumor, and invade the surrounding tissues (intravasation) becoming circulating tumor cells (CTCs). Cell motility is promoted through the interaction between tumor cells and immune cells. Then, CTCs spread throughout the circulatory system to the brain microvasculature (circulation) and after their adhesion with help of integrins, they start the extravasation across the blood–brain barrier (BBB). Tumor cells overexpress enzymes associated with mitogenesis, growth factors, metalloproteinases, and proteases allowing cell migration through the BBB. Once tumor cells are located in the central nervous system (CNS), an intense neuroinflammatory response is triggered. After extravasating, most tumor cells die or enter a state of dormancy (some of them could stay dormant at metastatic sites for long periods). Few tumor cells are able to proliferate within this new microenvironment and then form micrometastases and colonize the brain (colonization). The interaction between tumor cells and immune cells residing in the brain is critical for the establishment and growth of the tumor. COX2: prostaglandin-endoperoxide synthase 2; HBEGF: heparin-binding EGF-like growth factor; MMPs: metalloproteinases; VEGF: vascular endothelial growth factor.

To relocate to the CNS, disseminated circulating tumor cells (CTCs) must adapt to a microenvironment that is fundamentally different from the primary site. Immune cells, astrocytes, microglia, and neurons form a highly complex and dynamic TME, able to influence the survival of tumor cells and to modulate immune responses driven by metastatic brain cells [46,48]. The interaction between metastatic cells and the TME is critical for growth after cell seeding [23]. There is a complex reciprocal communication between metastatic tumor cells and their TME, which primes the successful outgrowth of cancer cells to form metastasis [45,49].

Astrocyte-derived exosomes mediate an intercellular transfer of *PTEN*-targeting microRNAs to metastatic tumor cells. As a consequence of PTEN loss, there is increased secretion of C-C motif chemokine ligand 2 (CCL2), which in turn induces recruitment of IBA1+ myeloid cells, enhances brain metastatic tumor cell proliferation, and reduces apoptosis [49]. The loss of BBB integrity is also a result of neuroinflammation and direct rupture of the barrier by tumor cells. Metastatic cells interact with neuroinflammatory cells and other components of the brain parenchyma, leading to tumor colonization. Secondary tissue colonization is a main bottleneck in metastatic development. Nonetheless, the pre-metastatic stage of the metastatic cascade remains poorly characterized. At the moment, studies using brain metastasis initiating cells (BMIC) show that the pre-metastatic stage of brain tissue colonization involves deregulated genes, many of which are active in invasive but not in proliferative mechanisms [50]. However, the process of metastatic brain colonization and changes in the microenvironment of metastatic tumors are not fully understood [23,51].

BMs, even in initial stages, are surrounded by a significant neuroinflammatory response mediated by activated astrocytes and microglia. Given the presence of the resident microglia and the lymphatic system, the brain is no longer considered a place with immunological privileges. The established metastases induce brain damage leading to the infiltration of immune cells, including CD8+ cytotoxic T lymphocytes. The expression of PD-1 and PD-L1 proteins in resected BMs indicates an immunosuppressive TME [23]. The extravasation of tumor cells, seeding, and formation of micrometastases is mediated by a combination of circulating proteins produced by tumor cells or cells in the TME. After extravasation, individual cancer cells are immediately surrounded by reactive astrocytes that act as an efficient first line of protection in the CNS by reducing the number of cells that initiate potential metastases. This natural defense contributes, in part, to the high inefficiency of colonization of the brain by cancer cells. Some cancer cells manage to survive and remain located in the perivascular niche next to the neural stem cells, where cancer cells have greater access to nutrients and oxygen, contact with the basal lamina of capillaries, and preferential access to angiocrine growth factors produced by endothelial cells [51].

The proliferation of cells that initiate metastases establishes a variable number of micrometastases. Some micrometastases can physically interact with reactive astrocytes. These interactions increase the growth of cancer cells and resistance to chemotherapy-induced apoptosis [51]. Astrocytes have also been shown to be critical modulators of immune responses in BM. They interact with inflammatory cells resident in the brain and are recruited along with

the microglia, leading to the establishment of an immunosuppressive microenvironment [49,52]. Thus, astrocytes are emerging as one of the main regulators of colonization and metastatic growth in the brain [46,53].

Several studies have shown that at every step during the metastatic cascade, cancer cells must engage different metabolic strategies, distinct from the primary tumor, to successfully metastasize [54,55,56]. While normal brain cells depend on glucose for energy production, metastatic cancer cells in the brain possess metabolic flexibility and depend not only on glucose for energy, but also on glutamine and acetate [57]. These metabolic adaptations are the result of interactions between cancer cells and brain cells including astrocytes and neurons, which promote rapid metastatic growth in the brain [57,58,59].

4. Molecular Determinants of Brain Metastasis in Lung Cancer and Their Implications for Treatment

Increasing evidence suggests that metastasis results from the aberrant activation of "invasive growth", a morphogenetic program that occurs during embryonic development and postnatal organ regeneration, driven by the *MET* proto-oncogene [60,61]. *MET* has been shown to play a central role in BM from lung cancer [62,63]. Additionally, Recepteur d'Origine Nantais (*RON*), also known as macrophage stimulating receptor 1 (*MSTR1*), a member of the MET family of receptor tyrosine kinases, harbors somatic mutations that are predicted to cause deleterious effects in BM from lung carcinoma [64]. Hyperactivation of the WNT/TCF signaling pathway has also been associated with BM formation in lung adenocarcinoma, mainly through the altered expression of the transcription factors HOXB9 and Lymphoid Enhancer Binding Factor 1 (LEF1), which stimulate tumor cell invasion and proliferation [65].

Studies have investigated metastatic genomic profiles in lung cancer. Genomic alterations in cancer-related genes in primary and matched metastatic tumors from 15 NSCLC patients, including 8 lung adenocarcinoma tissues [66], showed a concordance rate of 94% of recurrent alterations between primary tumor and matched metastasis, with *TP53* mutations being the most frequently observed [66]. Genomic characterization of stage IV lung squamous cell carcinoma of 79 patients reported hot-spot mutations in 8 main oncogenes (*EGFR, KRAS, BRAF, PIK3CA, NRAS, HER2, MEK1,* and *AKT1*) as well as exonic and intronic mutations of 279 cancer-related genes [67]. The data were also analyzed according to

two molecular subtypes: cases with fibroblast growth factor receptor 1 (*FGFR1*) amplification or mutation or loss of upstream phosphoinositide 3-kinase (PI3K) pathway genes, i.e., *PTEN* and *PIK3CA*. BMs were present in 11% (9/79) of cases, all from patients with PI3K altered tumors (27%; 9/33 patients). Six of the nine BM cases were further investigated by whole-exome sequencing (WES), RNA sequencing, and immunohistochemistry, and compared with a subset of four corresponding primary lung tumors. Results showed *PTEN* loss in 4/6 BMs and in all four primary tumors.

In addition, genetic alterations driving BM formation/progression were previously reported. Whole-exome sequencing of 73 BM cases from lung adenocarcinoma (BM-LUAD) identified *MYC*, *YAP1*, *MMP13* amplifications and *CDKN2A/B* deletions as pathogenic genomic changes [68]. Additionally, it was demonstrated that overexpression of these candidate driver genes (*MYC*, *YAP1*, or *MMP13*) promoted BM in patient-derived xenograft (PDX) mouse models [68]. In another study, by comparing focal somatic copy number alterations (SCNAs) in matched NSCLC-BM pairs, putative BM-driving genetic alterations were identified affecting multiple cancer genes, including several potentially targetable changes in genes such as *CDK12*, *DDR2*, *ERBB2*, and *NTRK1* [69]; these results were validated in an independent cohort of 84 BM samples and characterized SCNAs and mutations in *EP300*, *CTCF*, and *STAG2* genes, which play roles in epigenome editing and 3D genome organization [69]. Whole exome sequencing analysis of 12 paired primary NSCLC and matched BM have also identified BM-associated mutations in known cancer genes including *AHNAK2*, *ANKRD36C*, *BAGE2*, *KMT2C*, and *PDE4DIP* [70].

BMs may harbor high genetic heterogeneity and clonal differences between their corresponding primary tumors, suggesting that additional molecular changes may be acquired during metastatic progression [67]. Several studies have been performed in an attempt to address the question of clonality and molecular heterogeneity between primary tumors and brain metastasis from same patients. Some studies have collected and profiled metastatic lesions in an asynchronous mode with the primary tumor, allowing detection of evolutionary changes over time. In a report by Lee et al. [71], multi-omics sequencing of seven paired tumors and BM (collected at different time points) from patients with NSCLC, showed that 67% of mutations were common between metastatic and primary samples. In addition, these lesions had a similar tumor mutational burden (TMB). Further validation using publicly available data from a whole exome sequencing study of 35 BM and primary samples [72] showed 69% of shared mutations and similar TMB frequency. Based on these findings, the authors suggested

that metastatic events occur late during the evolutionary tumor development and progression cycles, likely upon the establishment of the majority of somatic mutations in the primary tumor [71]. Although the results of this study are based on a small sample set of 7 patients, the authors also suggested that a monoclonal mode of metastatic seeding may be predominant in most NSCLC cases.

Interestingly, Brastianos et al. [72] identified that, although there are genetic similarities between BM lesions arising in different brain sites as well as temporally separated, there is high genetic heterogeneity between BM and lymph node metastasis or extracranial distant metastasis. In addition, they reported actionable changes in BM, correlated with drug sensitivity to PI3K/AKT/mTOR, CDK, and HER2/EGFR inhibitors [72]. Other studies found similar results [73] and reported molecular changes likely selected during metastatic progression, such as deletions of *CDKN2A/B* which were common to metastatic and primary samples [68]. A recent whole exome sequencing study of 84 tissue samples from 26 patients compared genomic profiles of primary lung adenocarcinoma, liver and BM lesions; this study showed common driver mutations in *TP53* and *EGFR* in primary and metastatic samples. Additionally, a comparable TMB was present in all samples; however, the liver metastases had higher TMB and were more similar to the primary tumors than the BM lesions [74]. These authors also performed phylogenetic analyses and found that liver metastasis was genetically divergent from the paired primary tumors at a later stage of metastatic development compared to BM sites, suggesting that liver metastasis may arise preferably through a linear mode and BM may be established following a parallel mode of progression. It is important to highlight some differences among published studies, which may be due to different patient cohorts, distinct methodologies of sample collection with metastatic samples being collected either synchronously or asynchronously with the primary tumors, and different platforms of analyses. Although the current knowledge on the genetic divergence and phylogenetic evolutionary relationships among BM lesions and primary tumors, this is still an area that deserves further and detailed investigation.

Genetic studies have been performed to characterize BM. Examples include a targeted panel of 160 cancer-associated genes assessed in 39 lung adenocarcinoma patients with synchronous BM (n = 10, BM tissue only), metachronous BM (7/12 paired primary tumor biopsies and BM tissues) or extracranial metastases [75]. Results from this study showed aberrations affecting genes in the PI3K/AKT signaling pathway in primary and BM tissues. Comparing BMs versus extracranial metastases, *BCL6* and *NOTCH2* variants were the only

variants identified in at least four patients with BMs while appearing in only one or none of the non-metastatic cases. Unique variants were also detected in 20 genes (*TP53*, *SMAD4*, *SF3B1*, *NOTCH2*, *mTOR*, *MSH6*, *KRAS*, *KMT2D*, *KDM6A*, *IKZF1*, *GNAS*, *FANCD2*, *ERCC5*, *EP300*, *CREBBP*, *CDK12*, *BRCA2*, *BCL6*, *ATM*, and *ABL1*) in >33% of patients with BMs. Although there is a need for additional validation, this panel of genes was suggested to discriminate against the risk of developing BM [75]. More recently, tumor samples from 91 NSCLC patients (32 of which developed BM) were analyzed by Illumina RNA-sequencing. This study identified 22 genes (including *CELF1*, *NEURL2*, *CEBPB*, *AANAT*, *TMEM121*, *TWIST2*, *TNN*, *ST6GAL2*, *SLC38 A4*, *FFAR4*, *LRIG3*, *CYB561*, *DPP9-AS1*, *C5orf60*, *ZNF843*, *ANKRD62*, *ZNF439*, and *PSG2*) that specifically correlated with BM and not with metastasis to other sites [76].

NGS targeted sequencing of 416 cancer-associated genes in primary lung tumors (*n* = 61 patients: 50 adenocarcinoma, 3 squamous cell carcinoma, and 8 mixed histology) and paired BMs showed that mutations in *EGFR*, *KRAS*, *TP53*, and *ALK* were concordant between primary tumors and BMs in >80% of cases. Of these patients, 25 patients (41%) had synchronous BMs, which showed a larger number of cancer-associated mutations when compared to primary tumors; this was not observed for patients with metachronous BM [77]. These data suggest that synchronous BMs are likely to undergo genomic evolution with the activation of additional oncogenic mechanisms [77]. Another study with seven lung adenocarcinoma patients with BM identified that the protein expression levels of TYMS, CDK1, HJURP, CEP55, and KIF11 were highly predictive of BM in these patients [78].

Studies such as those outlined above show the existence of selective molecular mechanisms driving clonal evolution of BMs. Clones of metastatic cells growing in the brain evolve alternative routes, compared to other metastatic sites, mainly due to the hostile nature of the brain microenvironment for cancer cells. These data also have important implications to delineate tailored therapeutic approaches and to develop robust, clinically applicable biomarkers to identify patients at high risk for BM [23,51].

Effective treatments for patients with BM need to consider the molecular changes specific to metastatic cells, as well as the BM microenvironment [23]. Indeed, targeted therapies based on primary tumor driver mutations might fail to treat patients with BMs, due to the molecular divergence between BMs and primary tumors [34]. Current international recommendations include the identification of molecular alterations specific to BMs in tissues

and in liquid biopsies, which may be clinically applicable for early detection of BMs, and detection of BM-specific molecular changes to evaluate therapeutic response [34].

Considering the reported evolutionary changes and genetic differences between primary tumors and BM, as well as the molecular features that result from selective pressures of systemic treatments, there is a need for additional molecular profiling studies leading to biomarker development in BM. The relevance of novel biomarker discovery in BM and also extracranial metastasis includes the identification of molecular determinants/drivers of metastatic progression and the application of this knowledge for more effective treatment approaches to improve patient survival [79].

Recent studies applying single cell transcriptomic sequencing were able to unlock novel key molecular features of individual metastatic cells in lung cancer. Among these, Ruan et al. [80] characterized transcriptome changes in circulating tumor cells in cerebrospinal fluid of patients with lung adenocarcinoma leptomeningeal metastasis. They sequenced 792 transcriptomes of 5 patients and 3 controls and found a metastatic signature of genes with roles in metabolism as well as molecules related to cell adhesion. Furthermore, this study reported that there is higher heterogeneity among patients when compared to single cells isolated from the same patient [80]. In addition, Zhang et al. [81] identified differentially expressed genes between primary lung adenocarcinoma and BM isolated from two patient-derived xenografts (PDX). The authors suggested that *CKAP4* (Cytoskeleton Associated Protein 4), *SERPINA1* (Serpin Family A Member 1), *SDC2* (Syndecan 2), and *GNG11* (G Protein Subunit Gamma 11) are potential biomarkers to aid in prognosis assessment and therapy of patients with lung cancer BM [81].

Other technologies were applied for digital spatial RNA sequencing profiling of NSCLC and BM, and allowed a comprehensive assessment of biomarkers associated with primary and metastatic lesions, including analysis of the primary tumor immune and the brain microenvironments. Zhang et al. [82] analyzed a cohort of 44 patients with metastatic NSCLC using the NanoString GeoMx DSP platform. Among their findings, we highlight the highly immunosuppressive microenvironment associated with BM lesions compared to primary tumors, with reduced abundance of B and T-cells and higher infiltration of neutrophils. Their study shed light on the role of molecular changes in the tumor and BM microenvironments for establishment of the metastatic niche [82]. Studies such as these are relevant to determine clinically applicable biomarkers for patient treatment.

5. Non-Coding RNAs Play Important Roles in Brain Metastasis from Lung Cancer

Non-coding RNAs (ncRNAs) are regulatory molecules that modulate several biological functions, including gene expression, cell signaling, and genomic rearrangement [83,84]. NcRNAs are classified based on their lengths: small ncRNAs (sncRNAs; <200 nucleotides) such as microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs), and long noncoding RNAs (lncRNAs; >200 nucleotides) [85,86]. NcRNAs play roles in cancer progression, with specific expression patterns during metastasis development [87]. Alterations in ncRNA expression levels, mainly in miRNAs, could be associated with the development of BM in lung cancer since miRNAs have important regulatory roles in different steps of the metastatic cascade, including migration, invasion, adhesion, colonization, and epithelial-mesenchymal transition (EMT) [88,89,90]. MiRNAs can also contribute to disrupting the BBB [91] by creating a more hospitable environment for metastatic-initiating cells [92], establishing a pro-metastatic microenvironment [89,93] and modulating cancer stem cell (CSCs) properties that could contribute to the establishment of BM [94].

The mechanistic roles of oncogenic miRNAs in the development of BM from NSCLC have been explored. MiR-328 deregulation has been shown to promote BM in patients with NSCLC, partially by modulation of protein kinase C alpha (PRKCA), leading to high PRKCA levels and increased cancer cell migration [95]. MiR-378 has also been shown to be overexpressed and associated with an increased risk of BM and "brain-seeking" metastatic potential, due to its role in promoting cell migration, invasion, and tumor angiogenesis through modulation of *MMP-2*, *MMP-9*, *VEGF*, and suppressor-of-fused (*SUFU*) genes [96]. *SUFU* is involved in glioma cell growth and angiogenesis [97], and metastasis in lung adenocarcinoma [98]. Another report showed that miRNA-197 and miRNA-184 are overexpressed in *EGFR*-mutant BM when compared with *EGFR*-mutant primary tumors without BM [99]. However, this study did not include *EGFR* wild-type tumors from patients with and without BM for comparison with *EGFR*-mutant cases. Hence, more research is needed to understand how these miRNAs affect *EGFR* regardless of its status.

Tumor-suppressive miRNAs have also been implicated in metastatic progression. In animal models, miRNA-146a was suppressed in BM compared to primary tumors [100]. Overexpression of miR-146a suppressed the metastatic potential, including migratory and invasive activities, through upregulation of β-catenin and downregulation of heterogeneous

nuclear ribonucleoprotein C1/C2 (hnRNPC) [100]. In line with these findings, miRNA-95-3p is downregulated in BM of lung cancers compared to primary tumors [101]. Overexpression of miRNA-95-3p suppresses cyclin D1 expression through direct binding to the 3′ UTR of cyclin D1 mRNA and suppresses invasiveness, proliferation, and clonogenicity in in vitro assay [101]. Similarly, downregulation of miRNA-145 [102], and miRNA-375 [103], has been associated with BM formation in NSCLC; evidence suggests that while miR-145 overexpression reduces cell proliferation, there is no effect on the migration and invasion ability of cell lines. This indicates that miR-145 downregulation likely enhances cell proliferation after having reached the metastatic brain site, aiding in colonization, rather than in the early stages of metastasis [102].

Another miRNA found to be underexpressed in BM, compared to matched primary lung cancer tissues, is miRNA-768-3p [104]. Subramani et al. suggested that the brain microenvironment negatively regulates miRNA-768-3p, which enhances *KRAS* expression contributing to metastasis [104]. In another study with a cohort of 357 stage I NSCLC patients, 10 miRNAs correlated with BM (hsa-miR-450b-3p, hsa-miR-29c, hsa-miR-145, hsa-miR-148a, hsa-miR-1, hsa-miR-30d, hsa-miR-187, hsa-miR-218, hsa-miR-708, and hsa-miR-375) [105]. Taken together, these findings suggest that the loss of miRNAs with a tumor suppressive role could activate oncogenic pathways that are hallmarks of cancer, contributing to tumor development and progression to metastasis.

LncRNAs also play fundamental roles in lung tumorigenesis and metastasis [106,107]. They have been shown to modulate chromatin functions, control membraneless nuclear bodies' assembly and function, alter cytoplasmic mRNA stability and translation, and interfere with signaling pathways, depending on their localization and specific interactions with DNA, RNA, and proteins [108]. Examples of lncRNAs associated with lung cancer BM include the metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1* or nuclear enriched abundant transcript 2, *NEAT2*), which is overexpressed in a variety of tumors, including metastatic NSCLC [109]. *MALAT1* acts through promoting migration of cancer cells to the brain in an EMT-driven mechanism [109]. Furthermore, *MALAT1* promoted migration and invasion by targeting miR-206 and activating Akt/mTOR signaling in NSCLC tissues and cell lines [110]. Another lncRNA known in tumor development and progression, HOX transcript antisense RNA (*HOTAIR*), was associated with BM from NSCLC [111]. In vitro studies showed that *HOTAIR* enhances cell migration and anchorage-independent cell growth [111]. Nonetheless, the exact role and target of *HOTAIR* remains unknown.

Recently, it was also reported that the histocompatibility leukocyte antigen complex P5 (*HCP5*) is a potential driver for BM in lung cancer [112]. Computational bioinformatic analyses suggested that the ferroptosis-related competing endogenous RNA (ceRNA) *HCP5*/miR-17-5p/*HOXA7* axis may contribute to the development of BM in lung adenocarcinoma [112]. In addition, overexpression of AC122108.1 lncRNA promotes BM in lung adenocarcinoma through the Wnt/β-catenin pathway by directly binding to the aldolase A (ALDOA) protein; this mechanism enhances the proliferation, apoptosis, invasiveness, migration, and metastasis of lung adenocarcinoma cells [113]. In patients with limited-stage SCLC, a recent study using peripheral blood mononuclear cells (PBMCs) identified the low-level expression of lncRNA XR_429159.1 as a high-risk factor for BM [114]. However, the underlying mechanisms need to be further explored. Other lncRNAs involved in promoting metastasis in lung cancer include chromatin-associated RNA 10 (*CAR10*) [115] and brain cytoplasmic RNA 1 (*BCYRN1*) [116].

Recent studies have shown that lncRNAs are closely related to the increased permeability of the BBB in BM development and brain tumors [117,118,119,120]. In NSCLC, both exosomal-derived LINC01356 and lnc-MMP2-2 were found to increase BBB permeability and promote BM development. While the exosomal lncRNA LINC01356 remodels BBB by targeting cell junction proteins such as Occludin, Claudin, and N-cadherin [121], TGF-β1-mediated exosomal lnc-MMP2-2 may destroy the tight junctions of the BBB, thereby facilitating the passage of cancer cells [122].

Moreover, lncRNAs may interact with immune cells in the brain and contribute to a permissive environment for tumor growth [123]. For example, glioma cell-derived exosomes are able to transport lncRNA-ATB to astrocytes, promoting their activation, which in turn facilitates invasion and migration of glioma cells [124]. In breast cancer cells, loss of lncRNA X-inactive-specific transcript (XIST) triggers the polarization of microglia, resulting in increased expression of cytokines and suppression of T-cell proliferation [125]. Immune suppression is one of the mechanisms by which microglia promotes tumor progression in the brain [125]. Another study showed that JAK2-binding long noncoding RNA can promote breast cancer brain metastasis through a STAT3-dependent mechanism, which mediated recruitment of macrophages into the brain [126]. In addition, a recent report demonstrated that lncRNA (BMOR) is important for developing breast-to-brain metastasis by allowing tumor cells to evade immune-mediated killing in the brain microenvironment [127]. Altogether, these studies evidence the importance of lncRNAs for mediating communication between cancer

cells and the brain microenvironment. Given the importance of these lncRNAs, it would be interesting to explore whether it is also involved in lung-to-brain metastasis. **Figure 2** illustrates known miRNAs and lncRNAs involved in several important steps of BM development in lung cancer.

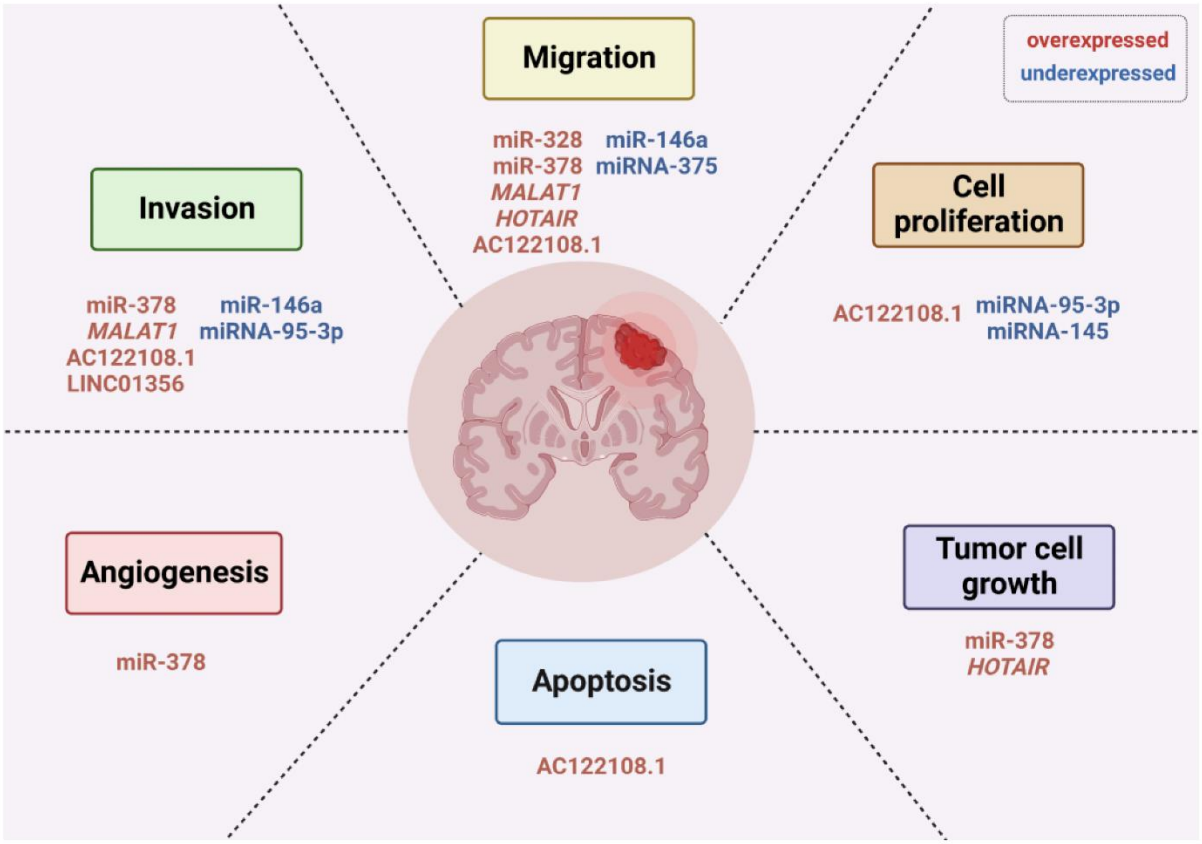

Figure 2. Non-coding RNAs (ncRNAs) and their roles in the hallmarks of cancer implicated in the development of BM from lung cancer. Red color indicates overexpressed ncRNAs; blue color indicates underexpressed ncRNAs.

Strategies that identify and target miRNAs and lncRNAs may be attractive as early diagnostic and therapeutic options. In recent years, the deregulation of ncRNAs in lung cancer has prompted preclinical studies examining the therapeutic potential of restoring and/or inhibiting such molecules [128]. The tissue-specific expression as well as high stability within body fluids makes them excellent candidates as biomarkers for diagnosis, prevention, and treatment of BM [128]. It has recently been demonstrated that miRNAs can be used to distinguish normal cells from cancerous ones and primary brain tumors from secondary brain tumors, as well as correctly categorize metastatic brain tumor tissues based on their expression

profiles [93]. These data indicate that miRNAs are promising candidates for clinical applications in BM. On the other hand, the roles and molecular mechanisms of many lncRNAs still remain elusive. Though promising, several challenges remain to be addressed to implement ncRNAs in clinical practice [93,129,130], such as the development of efficient delivery systems capable of crossing the BBB, with minimal toxicities, and the successful unloading of ncRNA therapeutics.

6. Advances in the Molecular Diagnostics of Brain Metastasis: Liquid Biopsies

Liquid biopsy is a new diagnostic concept based on the analysis of circulating tumor cells (CTCs), and/or molecules originated or secreted by tumor cells. Molecules derived from body fluids that are useful for liquid biopsy tests include circulating tumor DNA (ctDNA) and RNA (ctRNA), proteins, and microvesicles (e.g., exosomes) [131]. The ctDNA and ctRNA (coding and non-coding) are passively released from apoptotic or necrotic tumor cells, or are actively secreted by cancer cells [131].

Liquid biopsies are a minimally invasive alternative to tissue biopsies, particularly for tissues that are difficult to obtain, such as the brain. Furthermore, liquid biopsies can be serially repeated since they are minimally invasive and have low cost [132]. Liquid biopsies have an enormous potential to monitor treatment response, quantify minimal residual disease, and assess the emergence of clones resistant to therapy (**Figure 3**). Several types of body fluids are useful in the development of liquid biopsy diagnostic tests in cancer: blood, pleural effusion, and CSF [131]. Cancer-specific changes can be measured in liquid biopsies, including genomic, transcriptomic, proteomic, and CTC quantification. Translation of liquid biopsy tools into clinical practice is transforming diagnosis in oncology, as demonstrated by a large number of liquid biopsy diagnostic tests entering into the clinical setting [133]. Indeed, the first approved commercial liquid biopsy test detects *EGFR* mutations in ctDNA, and it is useful to select metastatic NSCLC patients for EGFR-TKIs [134,135].

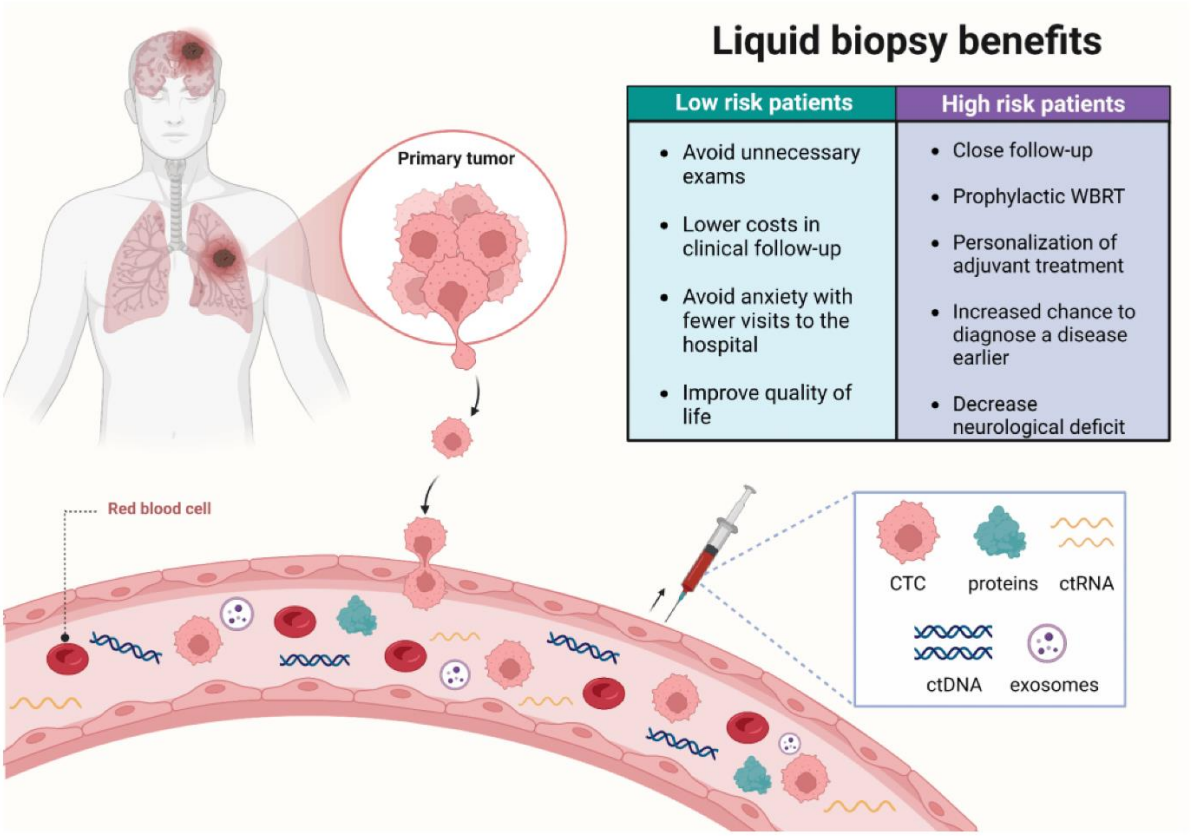

Figure 3. Clinical decisions and benefits for the patients upon using liquid biopsy tests to predict a low or high risk of developing brain metastasis. CTC: circulating tumor cell; ctDNA: circulating tumor DNA; ctRNA: circulating tumor RNA; WBRT: whole brain radiation therapy.

CTCs are isolated or clustered tumor cells released by the primary tumor or metastasis that leaks into the bloodstream and migrates towards the metastatic site. The frequency of CTCs is found on the order of 1–10 CTCs/mL of whole blood in patients with metastatic disease [136]. Different methods for enrichment, isolation, and identification of CTCs were developed according to their physical and biological characteristics. In lung cancer, isolation by size of epithelial tumor cells (ISET) was the earliest size-based method used for CTC detection, showing high sensitivity and reproducibility [137]. High levels of CTCs have been associated with worse outcomes in lung cancer. In 2017, Lindsay et al. evaluated the total number of CTCs as a prognostic marker in 125 treatment-naive patients with advanced NSCLC. Vimentin-positive CTCs were assessed according to treatment and the presence of *EGFR*, *ALK*, and *KRAS* mutations; a number higher than 5 CTCs was associated with reduced survival and an increase in vimentin-positive CTCs was associated with EGFR-mutated tumors, suggesting the presence of epithelial–mesenchymal transition characteristics [138]. Vimentin is a filamentous protein expressed in mesenchymal cells, and it is known to

maintain cellular integrity and provide resistance against stress [139]. It has been often recorded in cancers, including NSCLC and BM, as a marker of tumor cell invasion via its expression during the aberrant activation of epithelial–mesenchymal transition (EMT) [138]. During EMT, vimentin modulates genes for EMT inducers, as well as some key epigenetic factors. It suppresses cellular differentiation and upregulates their pluripotent potential by inducing genes associated with self-renewability, which increases the stemness of cancer stem cells, facilitating tumor spread and making tumor cells more resistant to treatments [140]. Vimentin overexpression has been associated with increased cancer cell growth, invasion, and migration, suggesting its potential application in cancer diagnosis and treatment [139]. Another study showed that the presence of CTCs was associated with low response rates, as well as shorter progression-free and overall survival, in patients with advanced NSCLC treated with both targeted- and chemotherapy [141]. CTCs derived from brain metastases were shown to have mutations in adaptive, cytoprotective genes with roles in the Keap1-Nrf2-ARE pathway, helping metastatic-initiating CTCs to survive in the blood circulation [142]. Therefore, CTCs may be an ideal source for determining the molecular portrait of metastasis where tumor biopsies are not clinically feasible. Importantly, CTCs can be expanded in vitro and in vivo, the latter by establishing CTC-derived xenografts as a means to characterize CTCs capable of initiating metastasis. Such applications have the potential to demonstrate the molecular mechanisms of metastasis initiation driven by CTCs and are promising in the discovery of novel molecular diagnostic and therapeutic strategies [143].

CTCs detected by liquid biopsies will help understand the molecular aspects of metastatic progression. Indeed, CTCs have been indicated as clinically applicable for many years, and a plethora of studies have demonstrated the correlation between CTC counts and metastatic disease in different cancer types [144]. In NSCLC patients with BM, CSF has been shown to be useful for CTC detection [145,146]. Recent functional studies have established in vitro and in vivo models from CTC-derived metastatic cells and are valuable to reveal molecular alterations specific to aggressive, metastatic-enabling clones [143]. Further development of methods for detection of metastasis-initiating CTCs will help elucidate the processes by which metastasis is established into the brain and extracranial sites. Darlix et al. [147] reported a prospective study for detection of suspected leptomeningeal metastasis in 40 patients with breast cancer. The authors tested the CellSearch® system, a clinically validated and FDA-approved test for CTC detection [147] and were able to identify CTCs in the cerebrospinal fluid of all cytology-positive samples. Interestingly, they detected CTCs in five

cytology-negative samples, demonstrating improved sensitivity of CTC detection using the CellSearch® system. Furthermore, they were able to detect HER-2 positive CTCs in the CSF of HER-2 negative tumors. This same system has been previously used to evaluate detection of both CTCs and exosomes in pancreatic cancer patients and was shown useful to accelerate diagnosis and treatment of surgically resectable cases [148]. Such studies highlight the importance of liquid biopsies as a potential tool to study molecular changes specific to more aggressive circulating tumor cells, as well as to refine molecular diagnostics and aid in treatment decisions that will impact patient survival [147]. Furthermore, the development of liquid biopsy-CTC-based biomarkers can be useful as a complementary tool to aid diagnostic imaging, augmenting early detection and, consequently, treatment intervention of occult BM [149].

Among cancer biomarkers, the proteome is a major source of circulating molecules that can inform clinically useful decisions [150]. A few examples include the circulating protein biomarkers CEA, PSA, β-hGC, CA 15-3, and CA 19-9 [151]. Carcinoembryonic antigen (CEA) is the most studied biomarker in lung cancer, investigated as a prognostic biomarker for BM development. High CEA serum levels were associated with BM in NSCLC [152]. CEA was shown as a prognostic biomarker for BM, as well as cytokeratin 19 fragments (CYFRA 21-1), cancer antigen 125 (CA125), cancer antigen 19-9 (CA19-9), and squamous cancer cell antigen in NSCLC [153]. Cancer antigen 125 has been used as a clinical tumor marker for prognosis and therapy monitoring in ovarian and breast cancer patients [154]. However, other studies reported CA-125 as a marker for worse prognosis in lung cancer [155] and a prognostic biomarker in BM [153]. In addition, high serum levels of lactate dehydrogenase (LDH), CEA, CYFRA 21-1, and CA125 were independently associated with BM in a large cohort of geriatric patients with lung adenocarcinoma [156]. Distinct from the genome, the proteome composition can change in response to variations in intracellular and extracellular conditions. Considering that gene expression implicates alternative splicing and post-translational modifications, the number of expressed proteins vastly outnumbers the number of genes. Therefore, proteome analysis can uncover molecular pathways, protein–protein interaction networks, and events underlying cellular phenotypes associated with the disease. The evolving field of oncoproteomics will likely derive novel biomarkers ready to use in liquid biopsies for clinical practice applications [151].

7. Treatment of BM from Lung Cancer

Primary management of BM predominantly has consisted of local treatments including surgery, stereotactic radiation or large field radiation therapy based on the knowledge of the heterogeneous penetration of systemic therapies into the brain. More recently, advances in systemic treatment were developed, particularly with the introduction of molecularly targeted therapeutics and immunotherapies. Nevertheless, direct evidence of systemic therapy in BM is limited since the presence of BM is generally an exclusion criterion in randomized trials, or patients with BM are underrepresented in these trials [157,158,159,160]. A comprehensive review of treatment for BM in patients with NSCLC is available by Tsui et al., 2022 [160].

Treatment for brain metastases comprises two broad categories, of symptomatic management and tumor-directed therapies [23]. Corticosteroids, such as dexamethasone, represent the main treatment for symptomatic patients, frequently prescribed in response to signs of increased intracranial pressure due to peritumoral edema [23]. Anticonvulsants may be prescribed to prevent seizures, and systemic steroids alone may improve neurological function and prolong survival by approximately two months [161]. Most therapies may include a combination of surgery (aiming at diagnosis and brain decompression), and/or adjuvant radiotherapy or systemic therapies. Other modalities include Stereotactic Radiosurgery (SRS) and whole-brain radiotherapy (WBRT) (**Figure 4**). The approach adopted for a given patient will depend on the performance status, and the distribution of intracranial and extracranial disease [162,163]. Novel methods for minimally invasive neurosurgery were demonstrated to have advantages such as inexpensive instruments, straightforward use and operation, and accurate positioning. Modern technologies for minimally invasive surgery are suitable for clinical practice in medical institutions [164].

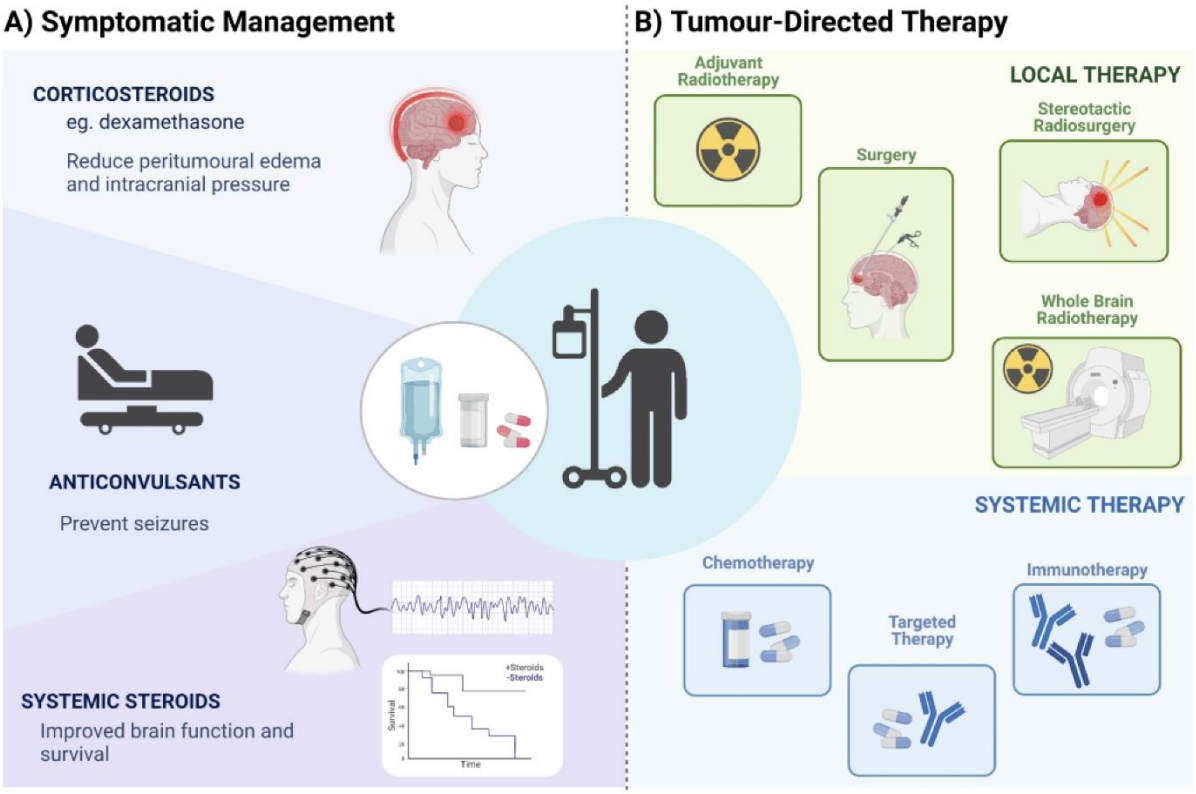

Figure 4. Summary of different strategies for the treatment of patients with brain metastasis including symptomatic management and tumor-directed therapies.

Corticosteroids have long been used to treat peritumoral edema. Their effects on improving symptoms are beyond the reduction of inflammation but include an upregulation of tight junction proteins (such as ZO-1 and occludin) [165], which are important in the maintenance of the blood–brain barrier's structure and function. Endothelial cells within and around the tumor are damaged by the presence of the tumor and the corresponding inflammation, leading to increased permeability of vessels and extravasation of fluid [166]. Despite the advantages of corticosteroid use, their symptom relief is temporary and dependent on the control of the local disease. In addition, the side effects of prolonged use are well known, such as diabetes, weight gain, cushingoid features, hypertension, myopathy, and osteoporosis—with increased risk of fractures [167]. Therefore, the recommended dose is the minimum needed to control symptoms, varying from 4 mg/day to 16 mg/day depending on the patient's symptoms [168].

The use of anticonvulsants for seizure prophylaxis is controversial. Despite being largely used in clinical practice, there is a paucity of high-level studies supporting their use. Current guidelines do not support the use of anticonvulsants for patients with newly diagnosed

brain tumors without a history of seizures. For patients undergoing surgical removal of tumoral lesions (which could be at potential risk of developing epileptogenic foci), there is insufficient evidence to support the use of anticonvulsants within the perioperative period [169]. In the case of seizures, the most commonly used agents are phenytoin, levetiracetam, valproic acid, and carbamazepine.

Surgical resection of BM may provide important benefits: symptom relief, de-obstruction of CSF pathways, dismissal of corticosteroid use, and samples for histopathologic and molecular analysis [19]. Indications for surgical resection include single or few intracranial lesions (up to three), large lesions, and accessible sites (i.e., sites where surgical corridors will not determine new deficits). En bloc resection seems to decrease the risk of future leptomeningeal dissemination and is preferable to piecemeal resection, even though larger tumors may require some intraoperative internal decompression in order to prevent additional neurological deficits [170,171]. Moreover, gross-total resection may improve overall survival [172]. Neuronavigation and intraoperative ultrasound are helpful for precise tumor localization. As with other brain tumor surgeries, brain mapping, awake craniotomy, and intraoperative monitoring are good options for safer resection.

Overall median survival rates after surgical resection of BM range from 9.8 to 24 months [173,174,175]. Within one year, survival rates are about 40–45% [173,176]. Factors determining better outcomes and prolonged survival are lower number of BM, lower age, better preoperative clinical performance, absence of extracranial metastasis, and association with other treatment modalities (i.e., radiotherapy and immunotherapy) [173,174,175,176,177,178]. For single BM, survival can be longer than two years in cases of complete resection and smaller tumor size [179,180].

Radiation therapy plays an important role in the management of BM. Post-surgical radiotherapy may reduce the risk of local recurrence, even though it is not clear whether it changes overall survival (probably because of initial poor clinical conditions) [181]. WBRT is commonly used for multiple lesions inaccessible for surgical resection. However, it carries a significant risk of neurocognitive decline and diffuse leukopathy. Therefore, stereotactic radiotherapy or stereotactic radiosurgery are preferable, as they target the lesions without causing diffuse brain damage [182,183].

The molecular characterization of lung adenocarcinoma was pivotal to patient management. However, this knowledge for SCLC and the squamous subtype of NSCLC had a

lower impact in patient treatment, mainly due to the low incidence or absence of targetable mutations in these tumor subtypes [159]. Current guidelines for BM treatment recommend targeted therapy only for those patients with oncogenic driver mutations [157]. Small molecule treatments have proven beneficial for palliative relief. For example, patients with NSCLC BM and positive for the *EGFR* mutation have shown meaningful CNS efficacy after treatment with third generation of EGFR TKIs such as icotinib [184] and osimertinib [185,186,187]. Similarly, intracranial response was observed in patients with BM treated with the third generation of TKIs targeting ALK rearrangements, alectinib [188], brigatinib [189,190], and ceritinib [191]. Other therapies have been explored for NSCLC, with BM showing promising results, such as capmatinib [192] and tepotinib [193], both targeting MET alterations; selpercatinib targeting RET fusions [194,195]; entrectinib [196], and larotrectinib targeting tropomyosin receptor kinase (TRK) fusion-positive tumors [197,198]; lorlatinib targeting *ROS1* [199]; and dabrafenib plus trametinib targeting *BRAF* V600E [200], among others [160,201].

The reduced penetration of chemotherapy agents through the BBB has limited its use as a primary treatment for BM in NSCLC [144]. Pemetrexed-cisplatin was shown to be effective for treatment of BM in patients with NSCLC with an objective response rate of 41.9% [182]. The FDA-approved drug Entrectinib was developed to target NTRK fusion as well as ROS and ALK tyrosine kinases and is capable of crossing the BBB. This drug has shown positive intracranial response rates in ROS-positive NSCLC [202,203], and NTRK fusion-positive solid tumors [196]. In addition, Lorlatinib, a potent brain-penetrant, third-generation tyrosine kinase inhibitor, has shown clinical activity in patients with advanced ROS1-positive NSCLC with BM [199]. Moreover, chemotherapy combined with immunotherapy has been shown to enhance the efficacy of immunotherapy, opening new windows for new first-line therapeutic strategies to benefit patients with advanced NSCLC [204,205].

Immunotherapy using ICIs is used in the management of NSCLC, particularly for patients without molecularly targetable disease. The benefit of ICIs in oncogene-targetable NSCLC is limited. Cumulative evidence suggests an interplay with tumor cell oncogenic signaling and tumor immunogenicity, leading to non-T cell-inflamed environment and resistance to ICIs. This complex interaction and balance between the TME and tumor cells triggers immune evasion mechanisms, including T cell exclusion, induction of regulatory T cells (Treg), and other immune suppressor cells, increasing PD-Ll expression, among others. For instance, poor efficacy of ICI monotherapy has been reported in patients with EGFR mutations as a consequence of characteristic low TMB and high expression of PD-L1 in these

tumors. Conversely, patients with ALK and ROS1 fusion-positive tumors present a relatively high prevalence of PD-L1 expression, but low TMB and short progression-free survival after monotherapy with ICI, indicating that subsets of NSCLC with EGFR and ALK/ROS1 positive mutations present minimal benefit from ICI despite high PD-L1 expression [206]. In contrast, KRAS mutated NSCLC presents high TMB, increased infiltration of lymphocytes PD-L1+, and an inflammatory tumor microenvironment, being more responsive to ICIs [207]. Therefore, NSCLC cases with distinct genomic subsets and specific oncogenic drivers show heterogeneous response to ICIs. To date, there is limited prospective data on the efficacy of ICIs therapy in NSCLC with driver mutations, mainly because ICIs clinical trials consistently exclude *EGFR*, *ALK*, and *ROS1* mutated tumors, thus precluding meaningful clinical information [208].

As previously acknowledged, patients with active BM are frequently excluded from clinical trials testing ICIs in NSCLC; therefore, the safety and activity of ICIs as a single-agent or combined with chemo or radiotherapy modalities are still under investigation [160,204]. A non-randomized, open-label, phase 2 trial showed that pembrolizumab provides similar response rates in intracranial and extracranial tumors, with improved overall survival in NSCLC BM presenting with PD-L1 expression ≥1% [209]. Therapy with pembrolizumab (anti–PD-1 monoclonal antibody) in patients with treatment-naive and previously treated PD-L1–positive advanced/metastatic NSCLC showed improved outcomes and fewer adverse events compared to chemotherapy alone in a pooled analysis of the Keynote-001, -010, -024, and -042 clinical trials, supporting the use of pembrolizumab monotherapy for these patients [210]. Additionally, Powell et al. [211] reported a pooled analysis of Keynote-021, -189, and -407 including 1298 NSCLC patients, of which 171 had baseline BM. In this study, patients with or without BM, treated with pembrolizumab plus platinum-based chemotherapy, showed improved clinical outcomes vs. chemotherapy alone across all PD-L1 positive samples [211]. In their study, patients with BM treated with pembrolizumab plus chemotherapy had a median overall survival of 18.8 months compared with 7.6 months with chemotherapy alone, and median progression-free survival of 6.9 and 4.1 months, respectively. Therefore, combined treatment regimens were suggested as a standard-of-care option for patients with advanced NSCLC, including those with stable brain metastases [211]. Similarly, the CheckMate 9LA, an international, randomized, open-label, phase 3 trial, showed that treatment with nivolumab plus ipilimumab combined with two cycles of chemotherapy resulted in superior overall survival when compared to chemotherapy alone, and suggested the use of this therapeutic

regimen as a first-line option in advanced NSCLC [212]. In a systematic review and meta-analysis, superior overall survival and progression-free survival was reported for patients with advanced NSCLC treated with ICIs compared to chemotherapy alone. This study also reported that a combination of treatment with nivolumab/ipilimumab plus chemotherapy resulted in further improved overall survival and progression-free survival of patients with BM [213].

Although the combination of radiation and targeted therapy or immunotherapy in the management of patients with BM NSCLC is controversial, clinical trials evaluating the role of local radiation with these therapies are ongoing (NCT04905550; NCT02978404; and NCT03916419, among others). Despite encouraging results with systemic therapy, the incidence of BM is still increasing. In addition, CNS progression and therapeutic resistance urgently require combinatorial strategies, including local therapy and novel CNS-penetrant drugs that can adequately treat intracranial metastases.

8. Conclusions and Perspectives

Approximately 40% of NSCLC patients develop BM during their disease course, leading to high morbidity and mortality rates. Management of patients with BM is challenging, and a multidisciplinary approach is necessary for treatment and disease control. In light of the increasing incidence of BM and poor clinical management, ongoing advances in multimodal treatments and targeted therapies are needed, including the development of CNS-penetrant agents that adequately target molecular alterations present in BM. In order to achieve effective and personalized treatment approaches for CNS metastases, -omics profiling should be integrated with microenvironment analyses. Research into the genetic variants and ncRNA expression may help stratify the lung cancer population by the risk of developing BM. Identifying the interactions between tumor cells and the brain microenvironment is also a key step in developing treatment strategies to block metastatic progression.

Improved treatment modalities have been implemented with the development of immune checkpoint inhibitors in combination with other systemic therapies. Although there is an observed gain in survival, patients with NSCLC and BM are still underrepresented in clinical trials, and there is a need for an assessment of routine MRI screening and biomarker identification.

The application of screening tools such as an MRI scan to identify patients with a higher risk of developing BM has the potential to improve patient outcomes. This is especially relevant since a proportion of patients with negative neuroimaging screens will develop a BM within one year of diagnosis; however, it remains controversial whether there is a need for routine neuroimage screens in patients with the early clinical stages of NSCLC.

Efforts for functional assessment of metastatic-competent cells have been described with in vitro and in vivo characterization of CTCs in liquid biopsies, as described in this review. Such studies are needed since the molecular mechanisms underlying the metastatic steps are not fully understood, mainly due to most studies focusing on brain lesions only, and not looking into isolating and identifying metastatic-enabling circulating cells. By integrating molecular analyses of BM collected at different time points during tumor evolution, researchers will aid in understanding disease progression in lung and other cancers. Furthermore, the application of advanced technologies, including single cell sequencing, will offer novel opportunities for analysis of CTC-derived metastasis, unlocking key transcriptomic and molecular changes associated with the metastatic cascade. Although high dimensional and more complex single cell sequencing analyses are still challenging, they hold potential for precision oncology in the context of complex and heterogeneous diseases including NSCLC-BM.

Therefore, it is urgent to fully elucidate the molecular mechanisms of BM, aiming at the development of successful therapeutic interventions, which will ultimately change the dismal prognosis of NSCLC patients with BM. Additionally, combined efforts to fully understand disease heterogeneity and metastatic evolution will lead to the development of better diagnostics for early detection of BM before clinical manifestation, improving patient outcomes and providing better chances of cure.

Author Contributions

P.P.R., S.A.D. and V.G.P.S. initiated the project. A.L.S., I.W.M., T.F.F., L.J.B., M.E.P., M.R.S., P.P.R., P.T.H.F., R.P.d.A., S.A.D. and V.G.P.S. designed, researched, analyzed, and wrote the topics covered in the article. L.J.B., M.E.P. and V.G.P.S. designed the figures. F.A.M., P.P.R. and W.L.L. reviewed and edited the manuscript. All authors contributed to data interpretation and manuscript preparation and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

References

The references used in this chapter will be presented at the end of thesis.

4.2 Capítulo 2

Identifying New Contributors to Brain Metastasis in Lung Adenocarcinoma: A Transcriptomic Meta-Analysis

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Simple Summary

Lung cancer patients have a high mortality risk due to brain metastases (BM). Understanding the molecular changes that contribute to BM is essential to identify potential therapeutic targets. Previous research has focused on primary tumor alterations, with less attention given to BM. This study examined a unique transcriptomic dataset assembled from previously reported RNA-seq, microarray, and single-cell analyses of BM samples from lung adenocarcinoma (LUAD) patients in pursuit of gaining a better understanding of the molecular landscape of BM. We found that dendritic cells and neutrophils were present in LUAD-BM, which could contribute to an immunosuppressive tumor microenvironment. The expression levels of 102

genes were altered, with *CD69* and *GZMA* identified as 'hub' genes, which could play a role in LUAD-BM. BM-specific gene expression was also observed, further supporting the presence of an immunosuppressive tumor microenvironment.

Abstract

Lung tumors frequently metastasize to the brain. Brain metastasis (BM) is common in advanced cases, and a major cause of patient morbidity and mortality. The precise molecular mechanisms governing BM are still unclear, in part attributed to the rarity of BM specimens. In this work, we compile a unique transcriptomic dataset encompassing RNA-seq, microarray, and single-cell analyses from BM samples obtained from patients with lung adenocarcinoma (LUAD). By integrating this comprehensive dataset, we aimed to enhance understanding of the molecular landscape of BM, thereby facilitating the identification of novel and efficient treatment strategies. We identified 102 genes with significantly deregulated expression levels in BM tissues, and discovered transcriptional alterations affecting the key driver 'hub' genes *CD69* (a type II C-lectin receptor) and *GZMA* (Granzyme A), indicating an important role of the immune system in the development of BM from primary LUAD. Our study demonstrated a BM-specific gene expression pattern and revealed the presence of dendritic cells and neutrophils in BM, suggesting an immunosuppressive tumor microenvironment. These findings highlight key drivers of LUAD-BM that may yield therapeutic targets to improve patient outcomes.

Keywords: lung cancer; bioinformatics; brain metastasis; immune cell; tumor microenvironment (TME)

1. Introduction

Brain metastases (BM) are a common and serious complication in patients with lung cancer, with tumors of the lung being the most prevalent cause of brain metastases [1,2,3,4,5]. The incidence of BM in lung cancer varies according to tumor histology where non-small-cell lung cancer (NSCLC), which accounts for approximately 85% of all lung cancer cases, has an incidence of BM ranging from 10 to 50% [6,7]. Lung adenocarcinoma (LUAD) is the predominant histological subtype of NSCLC, and represents approximately 40% of all lung

cancer cases. LUAD is frequently diagnosed at an advanced stage and characterized by the presence of metastases, with the brain being one of the main metastatic sites [8].

BM is associated with a wide range of symptoms, including headaches, seizures, and changes in vision, speech, and/or behavior [9]. These symptoms significantly impair patient quality of life, and the presence of LUAD-linked BM is associated with a dismal prognosis [10] and a median survival of approximately 15 months [10]. Treatment options for BM are limited but may include surgery, radiation therapy, and chemotherapy: ultimately, the choice of treatment depends on multiple factors including the patient's overall health and the extent of the metastasis [9,11].

Recent advances in targeted therapies and immunotherapies have shown promise in treating lung cancer, including BM. However, it is essential to conduct more research to improve outcomes since treating BM remains a persistently serious and difficult challenge [12,13,14,15]. Previous investigations have mainly focused on studying primary tumors with and without metastasis, in order to shed light on the underlying mechanisms of BM in lung cancer patients, paving the way for developing treatment strategies [16,17,18,19,20]. The molecular landscape of lung cancer-related BM has been recently reviewed comprehensively [11]. There is an urgent need for more research, since most studies are limited by small sample sizes and include patients with different disease subtypes lacking a complete description of patient clinical data. Therefore, further investigations are warranted, as the previous data have not been translated into clinical practice to provide discernible benefits to patients.

At present, targeted drugs available for the treatment of BM in lung cancer only benefit a subset of patients, are very costly, and associated with toxicity and development of resistance [12,13]. Therefore, it is of utmost importance to perform large-scale molecular studies of BM tissues to develop biomarker-based therapies. Genomic analyses of BM and corresponding primary tumors and other extracranial metastases have revealed that BM may harbor potentially actionable driver mutations [21]. The identification of specific molecular targets for BM will likely contribute to improved outcomes of patients who develop BM.

Here, we integrated data from different sequencing technologies (bulk RNA-seq, microarray, and single-cell RNA-seq) to provide a more comprehensive and detailed understanding of the molecular mechanisms and microenvironment components involved in BM from LUAD. In particular, we aimed to unravel transcriptomic changes that were specific to metastatic tumor cells within the brain, which might differ from the primary tumor.

Furthermore, we employed CIBERSORTx [22], a widely utilized analytical tool in tumor immunity research, to extract information on cell subsets from bulk gene expression data, with the objective of identifying potential biomarkers for BM and evaluating the presence of immune cells in LUAD patients' BM tissues. Finally, we sought to determine immune-related genes that could be applicable for diagnosing and treating brain metastasis by calculating the correlation between immune cells and 'hub' genes. This integrated approach holds promise in identifying novel therapeutic targets and fostering the development of more precise treatment approaches for this complex disease. A schematic overview of the methodology employed in our study is shown in **Figure 1**.

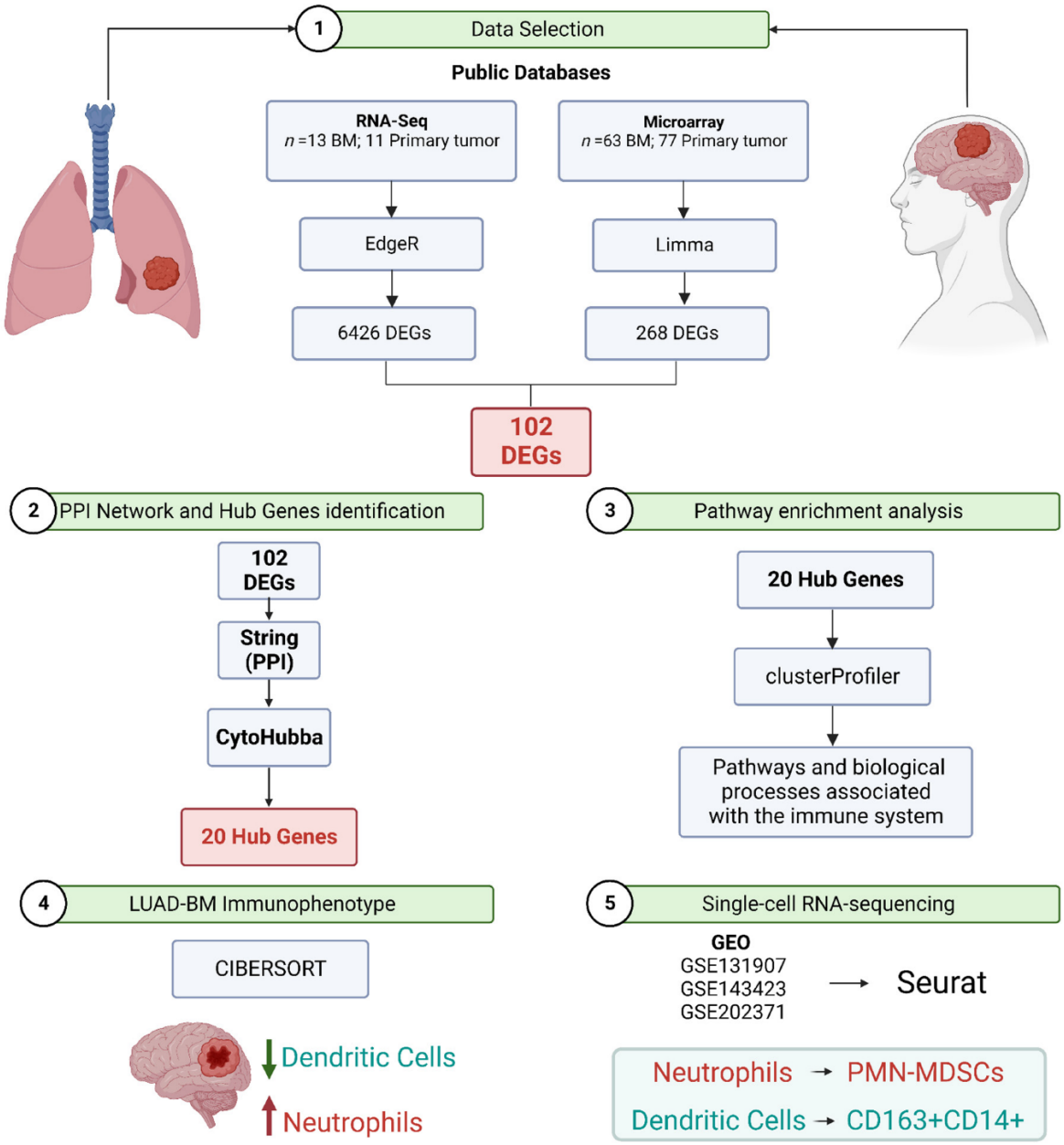

Figure 1. The experimental design and the significant findings of this study. BM: Brain metastasis; DEGs: Differentially expressed genes; PMN-MDSCs: Polymorphonuclear myeloid-derived suppressor cells; PPI: Protein–protein interaction.

2. Materials and Methods

2.1. RNA-Seq and Microarray Data Selection for Meta-Analysis

We searched for brain metastasis from lung adenocarcinoma-related RNA-seq and microarray datasets in different public repositories and databases using the following search terms: “brain metastasis”, “brain metastasis AND lung adenocarcinoma”, “brain metastasis AND transcriptome”, “brain metastasis AND transcriptomics”, “brain metastasis AND microarray”, “brain metastasis AND RNA-seq”. We included eight databases or repositories: Human Cancer Metastasis Database (HCMDB) [23], ArrayExpress [24], Restructured GEO (ReGEO) [25], European Genome-phenome Archive (EGA) [26], NCI Genomic Data Commons (GDC) [27], Sequence Read Archive (SRA) [28], The Database of Genotypes and Phenotypes (dbGaP) [29,30] and Gene Expression Omnibus (GEO) [31]. The search was performed on 12 September 2021. We selected the datasets using the following criteria: (1) gene expression data from BM tissue of patients diagnosed with LUAD as the primary tumor, (2) gene expression data from the primary tumor tissue of LUAD patients diagnosed with BM, (3) all BM locations were considered, (4) all platforms were considered. Exclusion criteria were: (1) leptomeningeal metastases, (2) studies that used only cell lines or animal models, and (3) review studies. The transcriptomic data used in this study were divided according to the sequencing technology: RNA-seq (n =13 BM; 11 Primary tumor), and microarray (n = 63 BM; 77 Primary tumor).

2.2. Identification of Differentially Expressed Genes Using RNA-Seq Data

The bam or fastq files were obtained from different repositories. The datasets obtained from SRA were downloaded using the SRA Toolkit (v.2.8.0) (available online at: https://www.ncbi.nlm.nih.gov/sra/docs/sradownload/, accessed on 1 October 2021) and converted from sra format to fastq using the fastq-dump --split-3 identifier. The datasets obtained from EGA were access-controlled; therefore, for each dataset, access was required through the Data Access Committees (DAC), providing documentation of the data access agreement. Once access was authorized, the bam files were downloaded using the EGA download client tool [32]. The arguments used were pyega3 -cf </Path/To/CREDENTIALS_FILE> datasets. The bam files were converted to fastq using Samtools [33]. The quality of the data was initially assessed with FastQC (v.0.11.9) [34] and summarized with MultiQC (v.1.10) [35]. All the reads were filtered by quality in SeqyClean

(v.1.10.09) [36] using Phred (QS) 30 and 30 for the mean and edge minimum score values and a minimum length of 65 bp. SeqyClean was also used to remove contaminant sequences from primers and vectors using the Univec database [37]. The reads were aligned with the Ensembl human genome assembly GRCh38 (release 99) using STAR 2-pass alignment method (v.2.7.8a) [38], and the expression count matrix was generated using HTSeq (v.0.11.1) [39]. Qualimap (v.2.2.1) [40] was used for quality control of the sequence alignment. Combat-Seq (v.3.36.0) [41,42] was used to remove batch effects between datasets. The EdgeR package was used to identify differentially expressed genes (DEGs) between BM and primary tumor. The *p*-value was adjusted by the Benjamini–Hochberg method to control the false discovery rate (FDR). Genes with the cutoff criteria of $|\log FC| > 2$ and adj. $p < 0.05$ were considered DEGs. DEGs were visualized as a volcano plot using the package (v.3.3.5) [43]. The gplots (v.3.1.1) [44], and biomaRt (v.3.13) [45,46] packages were used to build the heatmap and convert the ensembl_gene_id to hgnc_symbol, respectively.

2.3. Identification of Differentially Expressed Genes Using Microarray Data

Microarray data were obtained from the Gene Expression Omnibus (GEO) and ArrayExpress public databases. The E-MTAB-8659 dataset obtained from ArrayExpress based on the Illumina HumanHT-12 V4.0 expression beadchip platform included 63 brain metastasis samples from patients diagnosed with adenocarcinoma as the primary tumor. Additionally, we selected a GEO dataset (accession: GSE60645) that included 77 tissue samples from the primary LUAD tumor profiled using the Illumina HumanHT-12 V4.0 expression beadchip platform (there is no information about the presence or absence of BM in these data). Only datasets generated from the Illumina HumanHT-12 V4.0 expression beadchip platform were processed in order to minimize cross-platform variation. The microarray datasets were processed and normalized using *limma* (v.3.50.0). After normalization, *limma* [47] was used to identify DEGs between BM and the primary tumor. FDR value < 0.05 and $|\log FC| > 1.5$ were used as cutoff criteria for DEGs. The DEGs were visualized as a volcano plot using the ggplot2 package (v.3.3.5) [43]. The gplots package (v.3.1.1) was used for building the heatmap [44].

2.4. Identification of Differentially Expressed Genes (DEGs) Overlap between RNA-Seq Data and Microarray

To identify common DEGs between RNA-seq data and microarray data, only transcripts with HGNC-approved nomenclature were considered (available online at: www.genenames.org, accessed on 15 October 2021). The HGNC is responsible for approving unique symbols and names for human loci, including protein-coding genes, noncoding RNAs, and pseudogenes, to facilitate an unambiguous report. HGNC generally does not assign gene symbols to transcripts alternative or *splice* variants [48]. Genes common between both technologies were presented as a Venn diagram using the VennDiagram (v.1.7.1) [49]. Only the genes with consistent direction of expression change among the sequencing technologies were considered.

2.5. Functional and Pathway Enrichment Analyses

In order to obtain information about the biological function of the identified genes, we performed functional annotation and pathway enrichment analyses. To explore Gene Ontology (GO), we used the enrichGO() function from the R package clusterProfiler (v. 4.0.5) [50]. Additionally, we simplified the GO enrichment output by removing the redundancy of enriched GO terms using the simplify() function. The GO annotation file for the human species was obtained from the Gene Ontology (available online at: http://geneontology.org/, accessed on 1 October 2021) [51,52]. For the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, we used the enrichKEGG() function, also from clusterProfiler. The DOSE package (v.3.14) from Bioconductor was used for disease ontology enrichment analysis based on the Disease Ontology (DO) database (available online at: https://disease-ontology.org/, accessed on 1 October 2021) [53], as well as enrichment analysis based on the Network of Cancer Genes (NCG) database (available online at: http://ncg.kcl.ac.uk/index.php, accessed on 1 October 2021) [54]. For these analyses, the enrichDO(), and enrichNCG() functions were used, respectively. In all analyses, the *p*-value was adjusted using the Benjamini–Hochberg method to control the false discovery rate (FDR). Categories with a cutoff of $p.adj < 0.05$ were considered significant. Ggplot2 and GOplot packages were used to visualize the results [43,55].

2.6. Protein–Protein Interaction Network Construction for Selected Modules and Hub Genes Identification

Gene symbols for the common DEGs were converted to UniProt IDs using the org.Hs.eg.db (v.3.17) package [56]. Then, they were analyzed by the online tool STRING [57] for the construction of a Protein–protein interaction (PPI) network. Active interaction sources, including text mining, experiments, databases, co-expression, species limited to "Homo sapiens" and an interaction score > 0.4 were applied to construct the PPI networks. The results were visualized with CytoScape software (v.3.10.0) [58]. CytoHubba, a plug-in of CytoScape, was used to identify the PPI network's central elements [59]. Genes with the top 20 maximal clique centrality (MCC) values were considered 'hub' genes. The adjusted *p*-values (Benjamini–Hochberg method) were deemed significant at $p < 0.05$.

2.7. Immunophenotype of Brain Metastasis from Lung Adenocarcinoma

To estimate the immunological composition of the samples, we used the analytical tool CIBERSORTx [60]. CIBERSORTx includes the LM22 file, a signature matrix composed of 547 genes that distinguish 22 mature human hematopoietic populations, including seven types of T cells, B cells, plasma cells, NK cells, and myeloid subsets. Before being submitted to CIBERSORTx, the raw count data from RNA-seq was transformed into transcripts per million kilobases (TPM) using the convertCounts() function of the R package DGEobj.utils (v.1.0.4) [61]. The microarray data were processed by the *limma* package and used to validate the findings obtained with RNA-seq data. All samples were analyzed for immune cell profiles using CIBERSORTx with the number of permutations set to 1000 in order to obtain high statistical accuracy, and quantile normalization was turned off for RNA-seq data. The output files were downloaded as tab-delimited text files and imported into the R software (v.4.1.1) [62], which was used to identify differences in immune composition between BM and T. The normality test was performed using the shapiro.test() function. Differences were considered significant when $p < 0.05$ by the Wilcoxon–Mann–Whitney test. Then, we explored the association between the populations of immune cells in the studied groups; for this, the

Spearman correlation analysis was calculated using the rcorr() function of the Hmisc package (v.4.6-0) [63]. Additionally, we analyzed the correlation between the infiltration of the 22 cell types of the immune system and the expression of the 'hub' genes. The function chart.Correlation() from the PerformanceAnalytics package (v.2.0.4) [64] was used to obtain the expression scatter plots of the 'hub' genes along with Spearman correlation and estimated statistical significance. Values were considered significant when $p < 0.05$. Gene expression levels were determined with log2 TPM. The heat and chord plots were generated using the packages ggplot2 (v.3.3.5) [43], and circlize (v.0.4.13) [65].

2.8. Single-Cell RNA-Sequencing Data Processing and Analysis

Single-cell RNA sequencing (scRNA-seq) data for LUAD-BM samples were downloaded from Gene Expression Omnibus [31] (GSE131907, $n = 10$; GSE143423, $n = 3$; GSE202371, $n = 10$). Seurat (v.4.0.2) [66,67] was used for data quality control, integration, and analysis. Briefly, Seurat objects were created from individual expression matrices. Cells expressing <200 or >9000 genes (outliers) or with a percentage of mitochondrial genes higher than 10%, and genes expressed in less than 3 cells were all excluded (Figures S3 and S4). For the remaining cells, gene expression matrices were normalized using the NormalizeData function in the Seurat package. Seurat FindVariableFeatures were applied to select the top 2000 genes exhibiting the highest cell-to-cell variation. Gene expression matrices from different samples were then integrated. The batch effects were removed by canonical correlation analysis and mutual nearest neighbors-anchors using the functions SelectIntegrationFeatures, FindIntegrationAnchors, and IntegrateData. Subsequently, the integrated, batch-corrected expression matrix for all cells was scaled with the Seurat ScaleData function to apply a linear transformation. Principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) were used for dimensionality reduction. We determined the dimensionality of the dataset using the JackStrawPlot function. The top 15 principal components (PCs) were selected for dimensionality reduction. Before clustering the cells, a shared nearest neighbor graph based on the Euclidean distance in PCA space was conducted using Seurat FindNeighbors. Clustering was then performed with Seurat FindClusters with a resolution of 1.2. Marker genes for each cluster were determined with the Wilcoxon rank-sum test using Seurat FindAllMarkers. For each cluster, only genes that were expressed in more than 25% of

cells with at least 0.25-fold difference were considered. The annotations of cell identity on each cluster were defined by the expression of known marker genes.

3. Results

3.1. Datasets Selected for Meta-Analysis

We performed an extensive search in different public databases that contain transcriptomic data using microarray platforms and/or high-performance sequencing (RNA-seq). The search resulted in six studies: four reporting gene expression data from BM tissue of patients diagnosed with LUAD as the primary tumor; one study reporting gene expression data from primary tumor tissue of LUAD patients diagnosed with BM; and one study with both of the above information. The description of the publicly available studies is shown in **Table 1**. Dataset BioProject: EGAD00001007973 was excluded from our analyses as we were not able to access the raw data. Therefore, transcriptome expression data from five studies was included in our analysis, four with RNA-seq data and one with microarray data. Additionally, we selected a dataset (accession: GSE60645) based on the Illumina HumanHT-12 V4.0 expression beadchip platform that included 77 tissue samples from the primary LUAD tumors. The clinical information for the studies included in the meta-analysis is provided in **Supplementary Table S1**.

Table 1. Description of publicly available transcriptomic data used in this meta-analysis.

Study	Database	Access	Technology	Platform	Tissue	N° of samples	PMID
1	EGA	EGAS00001004078	RNA-Seq	Illumina HiSeq 2000	BM/T	5 BM 4 T	[68]
2	EGA	EGAS00001004006	RNA-Seq	Illumina HiSeq X Ten	T	7	[69,70]
3	SRA	PRJNA510710	RNA-Seq	Illumina HiSeq 2500	BM	2	[71]

4	GEO	GSE141685	RNA-Seq	Illumina HiSeq X Ten	BM	6	NA
5	ArrayExpress	E-MTAB-8659	microarray	Illumina HumanHT-12 V4.0 expression beadchip	BM	63	NA
6	GEO	GSE60645	microarray	Illumina HumanHT-12 V4.0 expression beadchip	T	77	[72]

EGA: European Genome-phenome Archive; GEO: Gene Expression Omnibus; BM: Brain Metastasis; T: Primary tumor. NA: Not available. *3 samples from the same patient (oligometastatic patient). N: number.

3.2. Integration of RNA-Seq and Microarray Datasets Identified 102 Differentially Expressed Genes in Brain Metastasis from Lung Adenocarcinoma

After quality control, mapping, and data normalization (Supplementary Figures S1 and S2 and Supplementary Tables S1–S3), we proceeded with differential expression analysis. A total of 164 samples (88 primary tumors and 76 BM) were included for differential expression analysis (**Figure 1**). Analysis was performed using the R programming language using two packages: *edgeR* and *limma*. Due to the large size of the tested genes, raw *p*-values were adjusted according to Benjamini and Hochberg's method for false discovery rate correction. The selection criteria were strengthened with a threshold of |logFC| > 2 and adj. $p < 0.05$ for RNA-seq and FDR value < 0.05 |logFC| > 1.5 for microarray data. These thresholds were chosen to detect significant gene expression changes against the inherent technical and biological variation within each platform. Volcano plots were generated to illustrate the distribution of each gene according to the logFC and adjusted *p*-value (**Figure 2**). In RNA-seq data, these parameters generated a list of 6426 differentially expressed genes (DEGs) in BM in comparison to the primary tumor, with 1850 upregulated and 4576 downregulated genes (**Figure 2**A) (**Table S4**). Within the microarray data, 268 genes were significantly differentially expressed in BM in comparison to the primary tumor, with 18 upregulated and 250 downregulated genes (**Figure 2**B) (**Table S5**). Interestingly, among the sequencing technologies, 106 DEGs (1.58%) (**Table S6**) were overlapping between RNA-seq and microarray, while 102 DEGs (1.52%) showed the same expression direction between the two sequencing platforms (**Figure 3**A) (**Table S7**). *JSRP1*, *CAMK1G*, *COX7A1*, and *NCALD* did

not show agreement in the direction of gene expression changes identified between sequencing technologies and were thus removed from subsequent analyses (**Figure 3**B).

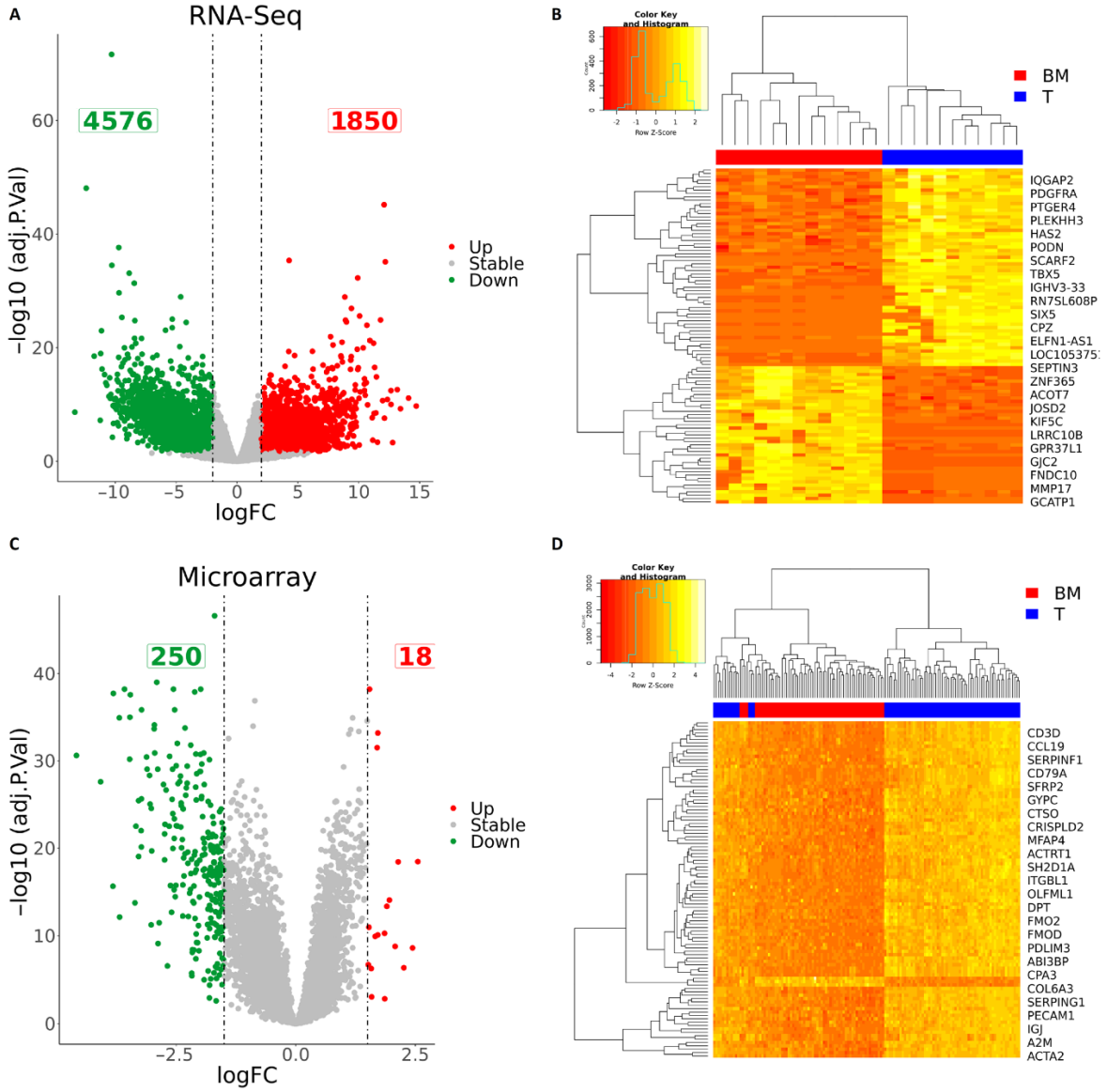

Figure 2. (**A**) Volcano plot indicating differentially expressed genes (DEGs) in brain metastases (BM) compared to the primary tumor (T) in RNA-seq data (red and green colors indicate |logFC| > 2 and adj. $p < 0.05$; other genes are colored gray. (**B**) Hierarchical cluster showing the expression profile of the top 100 DEGs in the RNA-seq data. (**C**) Volcano plot indicating differentially expressed genes (DEGs) in brain metastases (BM) in comparison to the primary tumor (T) in the microarray data (red and green colors indicate |logFC| > 1.5 and adj. $p < 0.05$; other genes are colored gray. (**D**) Hierarchical clustering showing the expression profile of the top 100 DEGs on the microarray data.

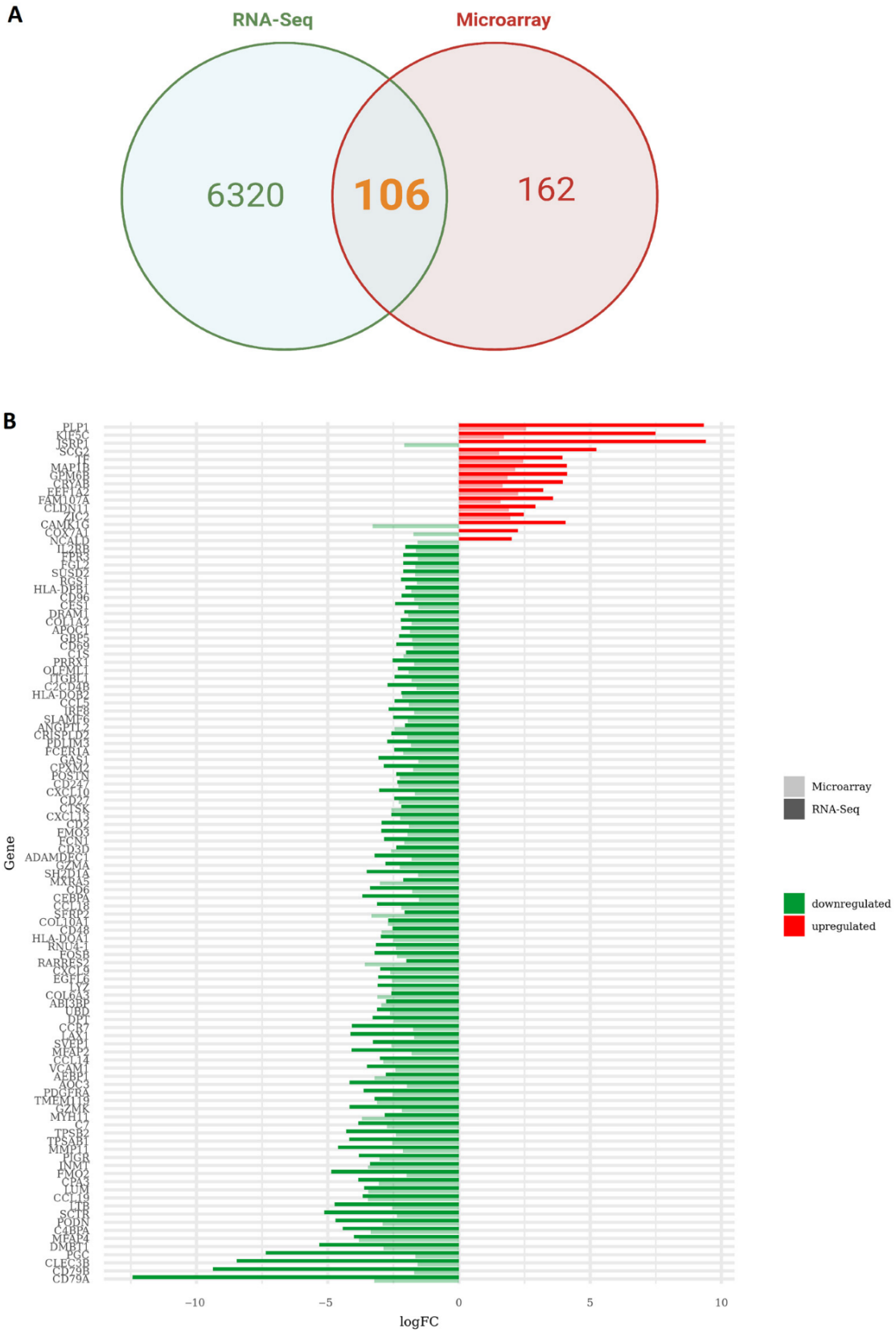

Figure 3. (**A**) Venn diagram showing the overlap between differentially expressed genes (DEGs) between RNA-seq and microarray data. (**B**) Bar graph indicating logFC values and expression direction of the 106 DEGs superimposed across sequencing technologies.

3.3. Pathway Enrichment Analysis Showed Pathways in BM Are Associated with the Immune System

To obtain information about functional mechanisms regulated by the 102 DEGs, analyses of functional annotation and pathway enrichment were performed. In the analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, enrichment of the following molecular pathways was identified: cell adhesion, chemokine signaling, cytokine–cytokine receptor interaction, and Th1, Th2, and Th17 cell differentiation pathways (**Figure 4**A) (**Table S8**). The dysregulated expression of these genes may play a crucial role in modulating immune responses and cell-to-cell communication processes. Furthermore, functional enrichment analysis was conducted to predict the biological functions associated with the DEGs. This analysis identified biological processes related to the immune response, chemokine response, and extracellular matrix organization. These findings suggest that the DEGs may be involved in immune-related processes. Moreover, the enrichment of cellular component terms mainly associated with the cell membrane indicates that the DEGs may have important roles in membrane-associated functions and signaling processes (**Figure 4**B) (**Table S9**).

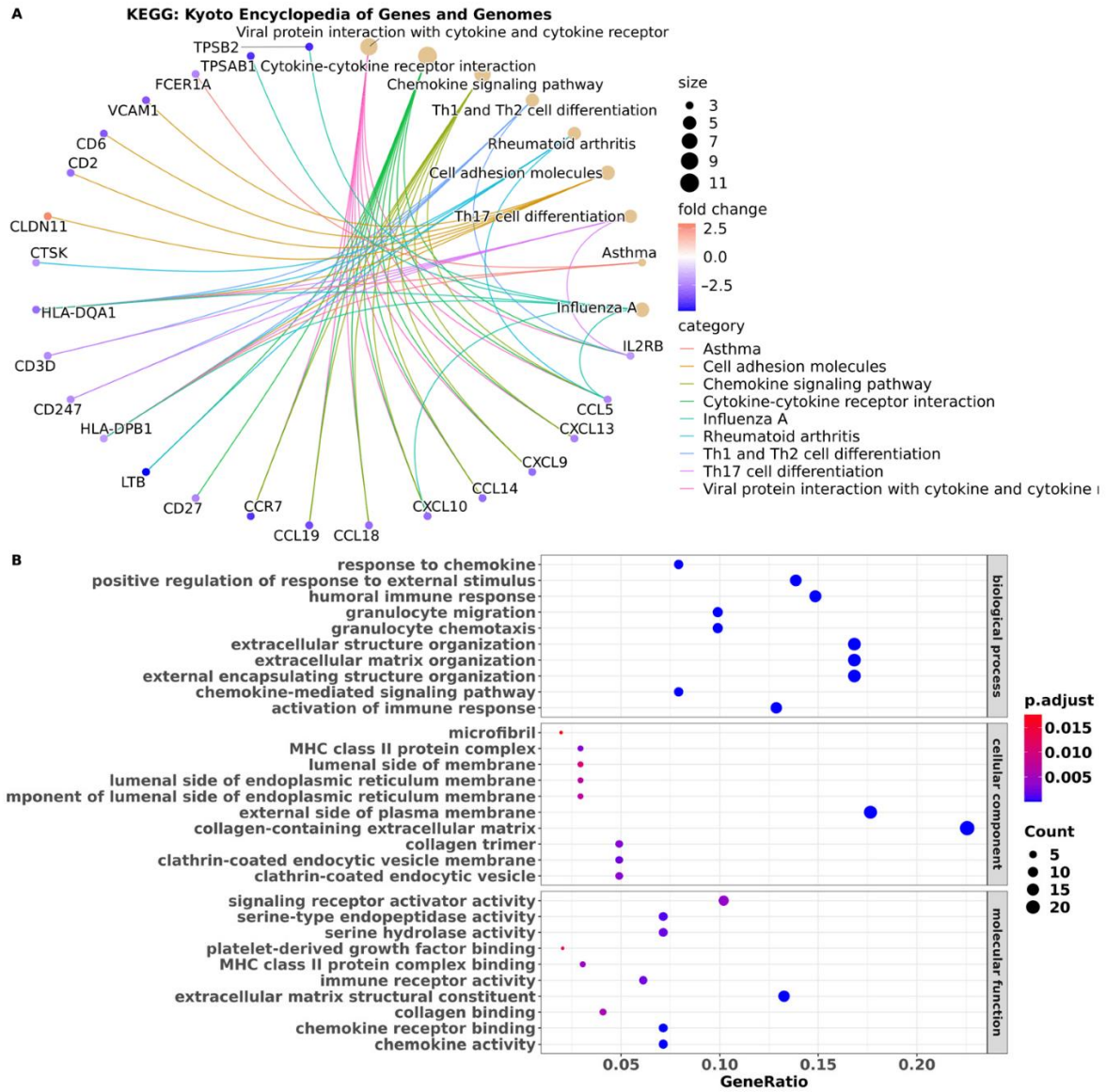

Figure 4. Functional annotation and enrichment analysis. (**A**) Enrichment analysis interaction network from the Kyoto Encyclopedia of Genes and Genomes (KEGG). The node size represents the number of genes according to each KEGG category, and the color of the nodes represents the logFC value per gene within each enriched KEGG category, as shown by the legend. Borders highlight interactions between the KEGG category and the genes that enrich it. (**B**) Enrichment dot plot of the term Genetic Ontology. The graph presents the top 10 enriched ontologies for each of the instance terms (biological process, molecular function, and cellular component) with adj. *p*-value < 0.05. The *X*-axis presents the number of genes that enrich the ontology term, and the point size is proportional to this number.

3.4. Brain Metastasis from Lung Adenocarcinoma Exhibits Distinctive Characteristics That Distinguish It from All Other Types of Cancer

In order to gain insights into the functional mechanisms regulated by the DEGs, we conducted analyses of functional annotation. One aspect we explored was the relationship of

the DEGs in the context of diseases. To achieve this, we utilized the DOSE package [**73**] for disease ontology enrichment analysis. Disease ontology provides a framework for annotating human genes within the context of specific diseases, facilitating the translation of molecular findings into clinical relevance. Using gene set enrichment analysis, we identified significant associations between the DEGs and interstitial lung disease. Specifically, out of the 102 DEGs, nine genes (*CTSK*, *COL1A2*, *CCL5*, *AEBP1*, *CXCL9*, *CXCL10*, *CCL18*, *PDGFRA*, *CCL19*) exhibited significant associations with this condition (**Figure 5**A) (**Table S10**). We also identified significant associations between the DEGs and bacterial infection disease (*TF*, *HLA-DPB1*, *CD247*, *CCL5*, *CD27*, *HLA-DQA1*, *GZMA*, *CXCL9*, *CXCL10*, *VCAM1*, *SH2D1A*, and *CCR7*).

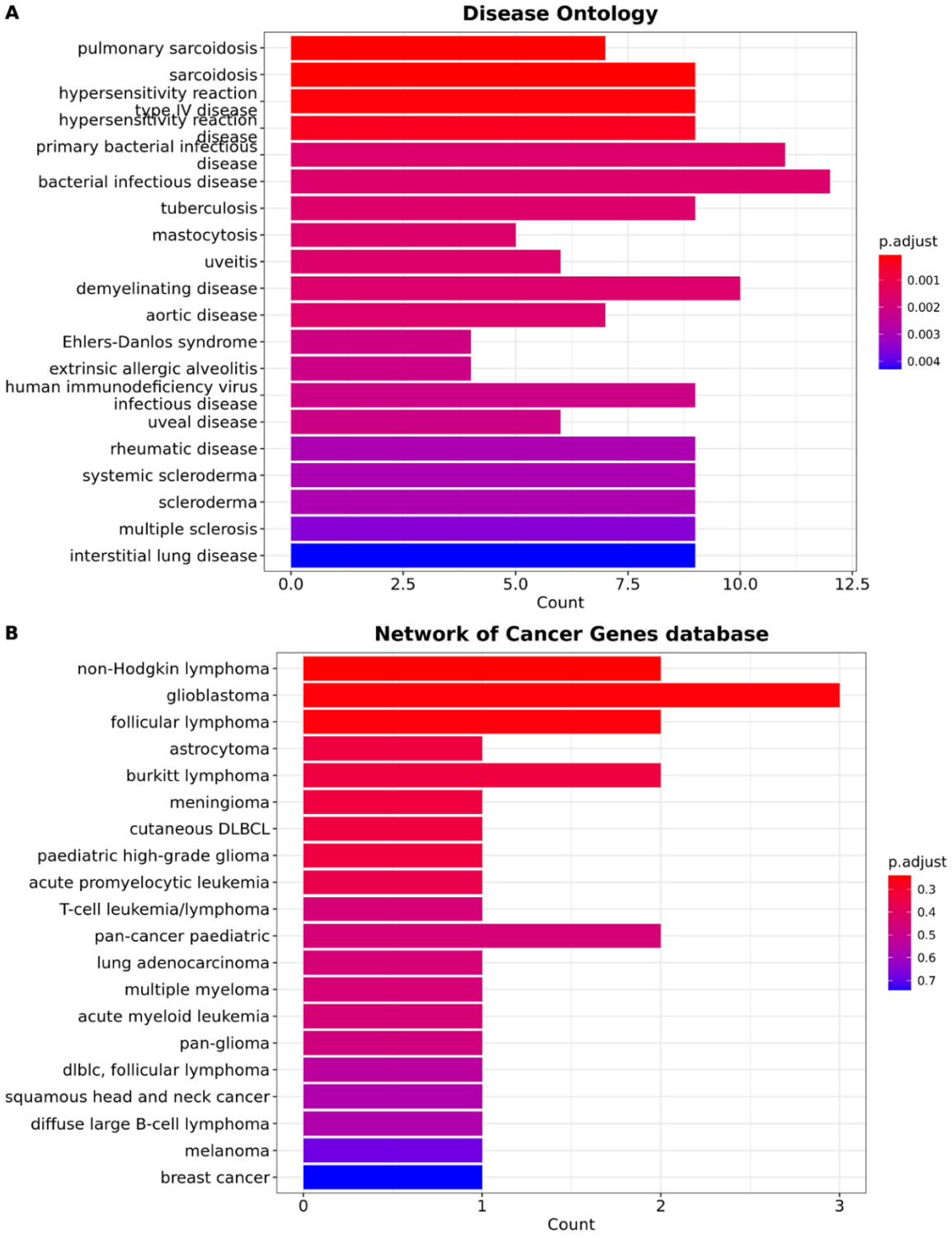

Figure 5. Gene set enrichment analysis. (**A**) Bar plot of Disease Ontology enrichment analysis. The plot was created using clusterProfiler and Disease Ontology annotations from the DO database. The *x*-axis represents the number of genes enriching the ontology term, and the color of the bars represents the adjusted *p*-value. (**B**) Bar plot of enrichment analysis based on the Network of Cancer Genes database. The *x*-axis represents the number of genes enriching the ontology term, and the color of the bars represents the adjusted *p*-value.

Additionally, we performed an enrichment analysis based on the Network of Cancer Genes database to further explore the relationship between the DEGs and specific types of cancer. Surprisingly, our results did not reveal any significant associations between the DEGs and particular cancers, indicating that metastatic brain tumors possess unique characteristics that distinguish them from other types of cancer (**Figure 5**B) (**Table S11**). These findings highlight the distinct molecular features and underlying mechanisms of metastatic brain tumors.

3.5. Protein–Protein Interaction Network Constructed from DEGs Reveals the Biological Network of Brain Metastasis from Lung Adenocarcinoma Is Associated with the Immune System

In order to understand the functional relationship of DEGs and the biological phenomena involved, we investigated the functional interactions of the proteins encoded by these genes through the construction of a connectivity network using the database STRING. This tool allows for achieving a comprehensive and objective global network, including direct (physical) and indirect (functional) protein interactions. The network of genes related to BM (**Figure 6**A) has 101 nodes. The network nodes represent proteins (each node represents all proteins produced by a single protein-coding gene locus) and 279 edges (edges represent protein–protein associations). Regarding the centralities of the network, the network presents the average of the local clustering coefficient = 0.509 and average degree = 5.52. The clustering coefficient is a measure of how connected the network nodes are. Highly connected networks have values close to 1. The average degree of a node is the number of how many interactions a protein has on average in the network and indicates the regulatory relevance of this protein. The PPI enrichment value was $p < 1.0 \times 10^{-16}$. This indicated that the proteins are biologically significantly connected. The interaction with the highest combined score was between the CD3D and CD247 proteins (combined score = 0.999) (**Table S12**). The combined score is a confidence indicator, that is, the probability that the STRING considers an interaction to be true, according to the evidence available. All scores are ranked from 0 to 1, with 1 being the highest possible confidence.

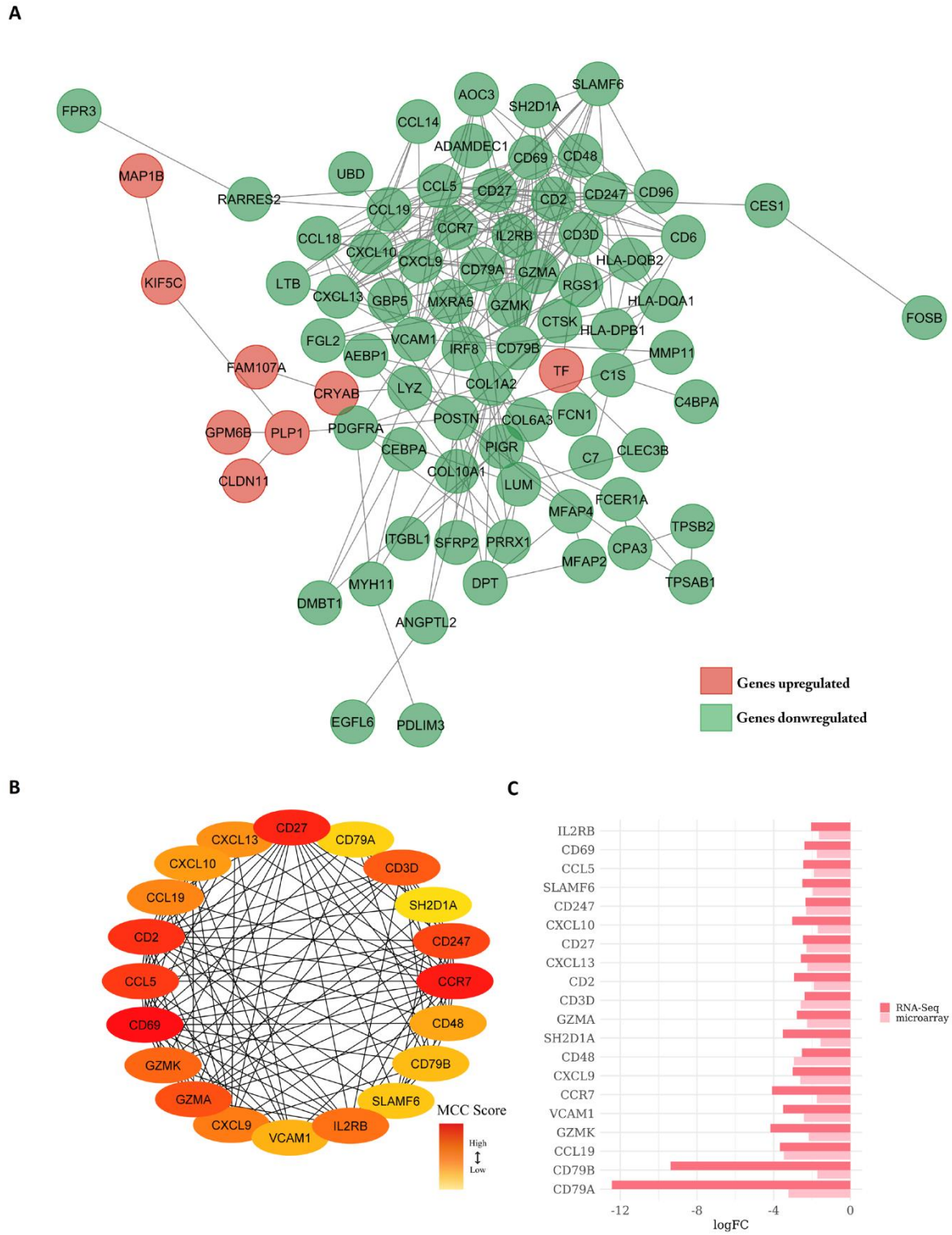

Figure 6. (**A**) Protein–protein interaction network (PPI) visualized with Cytoscape. The nodes represent the proteins. Borders highlight interactions between proteins. Upregulated genes are marked in red and downregulated genes are marked in green. (**B**) 'Hub' genes. The color red to yellow represents the degree of connectivity from top to bottom. (**C**) Bar chart indicating logFC values and expression direction of the 20 'hub' genes.

After building the PPI network, we built a co-expression network using the maximal clique centrality (MCC) algorithm from the CytoHubba plug-in from Cytoscape. The tool allows for inferring the importance of nodes and helps to identify the central elements of a biological network. The MCC method classifies nodes (proteins) into high- and low-grade categories. The protein grade is a measure that indicates the degree of correlation between the protein and the essentiality of its corresponding gene, i.e., proteins with higher grades are more likely to be essential proteins in the biological network. Based on this analysis, we identified 20 key network elements (**Figure 6**B), referred to as 'hub' genes. The 'hub' genes and the score grades are shown in **Table S13**. Among these, the CD69 gene showed the highest degree of connectivity (score = 396,192). Notably, all of these genes were downregulated (**Figure 6**C).

Related to the 'hub' gene enrichment analysis, key enriched terms of the biological process include T-cell activation, chemokine response, and upregulation of cell–cell adhesion (**Table S14**). For enrichment analysis of cell components, the results are related to cell membrane components. The corresponding molecular function terms are represented in related terms, mainly chemokine activity (**Table S14**). In the enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, the main pathways identified were T-cell activation, Th1 and Th2 cell differentiation, and the TNF signaling pathway (**Table S15**).

3.6. The Fraction of Neutrophils Is Greater in Brain Metastasis Compared to the Primary Tumor

Considering that the tumor immune microenvironment is known to play an important role in metastatic progression, and that our DEG analysis revealed enrichment of immune-related pathways and processes in LUAD-BM, we investigated the composition of immune cell infiltrates in the BM samples using the CIBERSORTx algorithm. **Figure 7**A summarizes the results obtained from the analysis of 13 BM samples and 11 primary tumors (RNA-seq data). In both groups, resting memory CD4 T cells comprised the largest cellular fraction of the total immune cells, with 17.4% of the total immune cells in BM and 15.24% in primary tumors (**Figure 7**A). After estimating the composition of immune cell infiltrates, we identified significant variations between the studied groups. We found that the fraction of resting dendritic cells (also referred to as immature dendritic cells) was significantly lower in BM

compared to the primary tumor (T); while the neutrophil fraction was higher in the BM compared to the primary tumor (T) (**Figure 7**B,C).

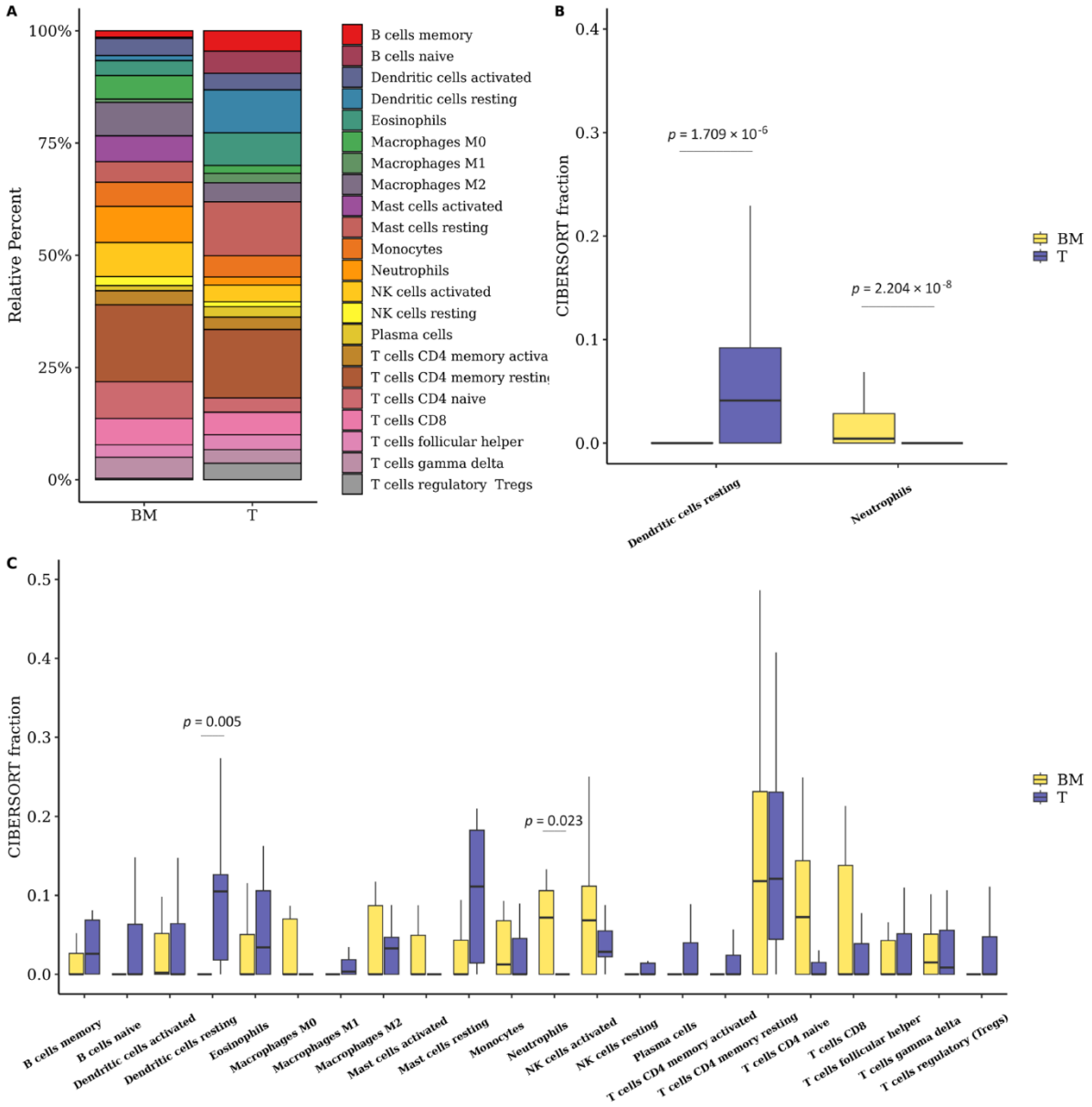

Figure 7. (**A**) Infiltrating immune cell composition in brain metastasis (BM) and primary tumor (T) is summarized from mean values calculated for each group. The bar graph shows the difference between CIBERSORTx immune cell fractions between brain metastases (BM) and primary tumors (T). (**B**) Results were generated using microarray data. (**C**) Results were generated using RNA-seq data.

We also explored the correlation between 22 immune cell subtypes in BM and the primary tumor by Spearman's correlation (**Tables S16 and S17**, respectively). We identified

several highly positive relationships between infiltrating immune cells in BM samples, while the mutual relationship between immune cells was reduced in primary tumor samples (**Figure 8**A,B). In BM, the highest positive correlation was between follicular T helper cells and plasma cells (Rho = 0.77, *p*-value = 0.001); while in primary tumors, the highest positive correlation was between plasma cells and monocytes (Rho = 0.80, *p*-value = 0.002). We further explored the correlation between the infiltration of the 22 cell types of the immune system and the expression of the 20 previously identified 'hub' genes in the BM samples (**Figure 8**C). The *CD27*, *CXCL13*, and *CD79B* were the genes that showed the highest number of correlations between their expression and the infiltration of immune cells, a total of seven significant correlations were identified for each of these genes. All genes were significantly correlated with the expression of CD8 T cells, naive CD4 T cells, monocytes, M1 macrophages, and resting mast cells; all correlations were positive except for resting mast cells (Rho = −0.77; −0.65 and −0.63, *p*-value < 0.05) and naive CD4 T cells (Rho = −0.56; −0.76 and −0.68, $p < 0.05$). Related to immune cells, CD8 T cells, naive CD4 T cells, regulatory T cells, and monocytes were the cell subtypes that showed the highest number of correlations significant with the expression of the 'hub' genes. Monocyte infiltration was correlated with the expression of 18/20 'hub' genes (*CD69*, *CCR7*, *CD27*, *CD2*, *CCL5*, *CD247*, *GZMA*, *CD3D*, *GZMK*, *IL2RB*, *CXCL9*, *CCL19*, *CXCL13*, *CXCL10*, *CD48*, *VCAM1*, *CD79B* and *SLAMF6*), followed by CD8 T-cell infiltration correlated with expression of 16/20 'hub' genes (*CD69*, *CD27*, *CD2*, *CCL5*, *CD247*, *GZMA*, *CD3D*, *GZMK*, *IL2RB*, *CXCL9*, *CCL19*, *CXCL13*, *CXCL10*, *CD48*, *CD79B* and *SLAMF6*). Naive CD4 T cells and regulatory T cells were both correlated with the expression of 15 'hub' genes ($p < 0.05$). Therefore, 'hub' genes were correlated with immune-infiltrated cells in BM from LUAD.

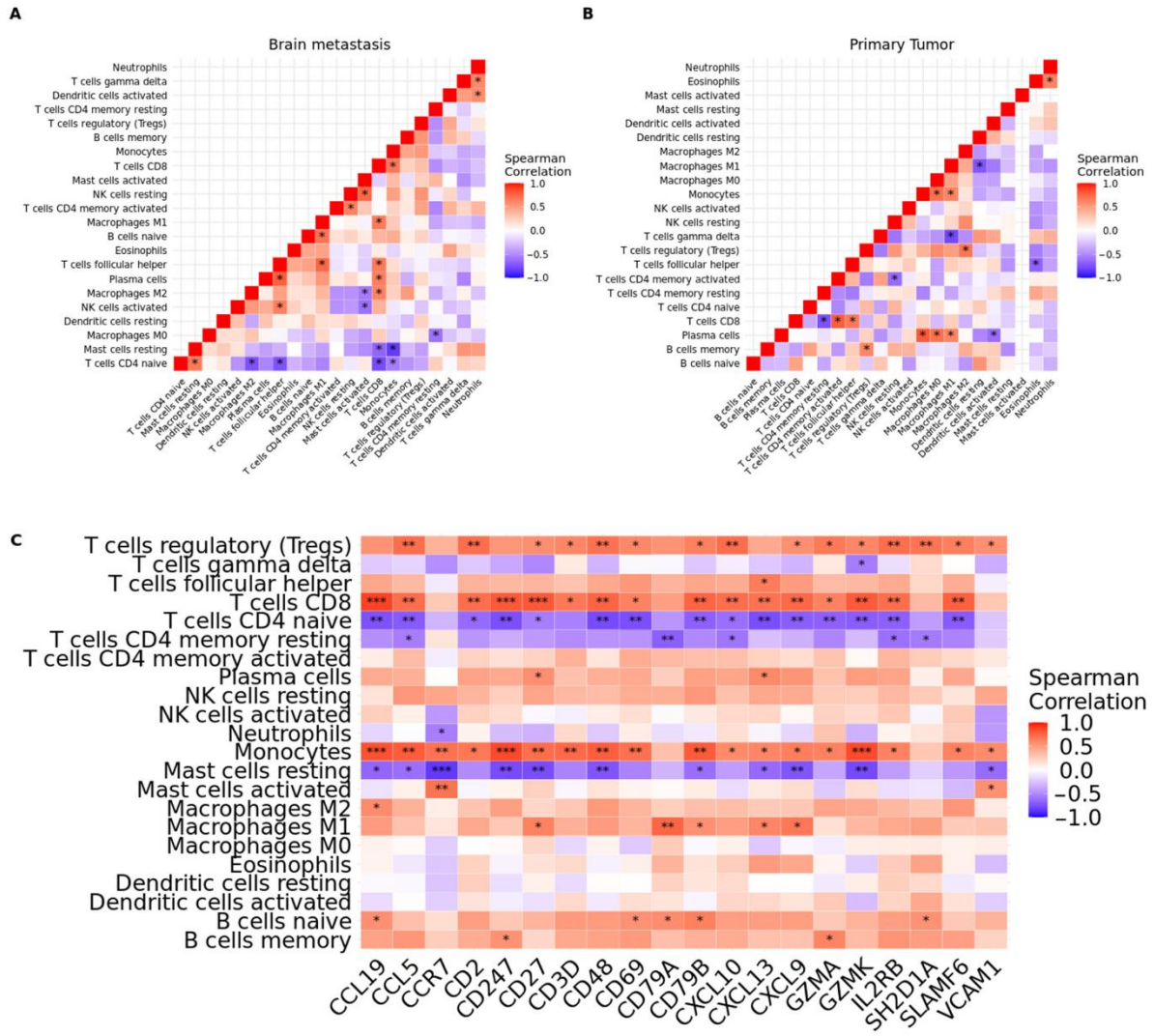

Figure 8. Correlation matrix of all 22 immunological proportions. (**A**) Brain metastasis. (**B**) Primary tumor. * represents significant correlations ($p < 0.05$). (**C**) Correlation plot (Spearman correlation coefficients) of 'hub' gene expression and proportion of infiltrating immune cells in brain metastasis. Colors in the heatmap indicate the strength of the correlation. Asterisks indicate the level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.7. scRNA-Seq Data Reveals an Immunosuppressed Tumor Microenvironment in BM from Lung Adenocarcinoma

To explore the function of neutrophils and dendritic cells in LUAD-BM we obtained a total of 57,774 cells from three different datasets (GSE131907, n = 24,508 cells; GSE143423, n = 12,196 cells; GSE202371, n = 21,070 cells) (**Figure 9**A). We applied the uniform manifold approximation and projection (UMAP) method and successfully classified the cells into 43 separate clusters (**Figure 9**B). Sub-clustering of 13,427 dendritic cells was

identified, as shown in **Figure 9**C (**Table S18**). For a more comprehensive analysis, we reclassified DCs into six subsets using markers previously described [**74**,**75**]. These subsets included CD1c+ DCs (Langerhans cells, LCs), CD141+ DCs, CD207 + CD1a+ LCs, pDCs (plasmacytoid DCs), CD163 + CD14+ DCs, and activated DCs (**Figure 4**D). Interestingly, a subset of activated DCs was not identified (**Figure S5**), only CD163 + CD14+ DCs were found (**Figure 9**D). The cluster of neutrophils containing 3762 cells was identified as polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) (**Figure 9**). We next carried out a pathway analysis which showed that PMN-MDSCs was enriched in the IL-17 signaling pathway and NF-kappa B signaling pathway (**Figure 9**E) (**Table S19**). Then, we found that CD163 + CD14+ DCs showed increased expression of HLA genes and antigen processing and presentation pathway (**Figure 9**F). They also showed high expression of *CCL3*, *CCL2*, and *CXCL3* (**Table S20***),* which might be involved in the recruitment of activated T cells to inflammation sites [**76**].

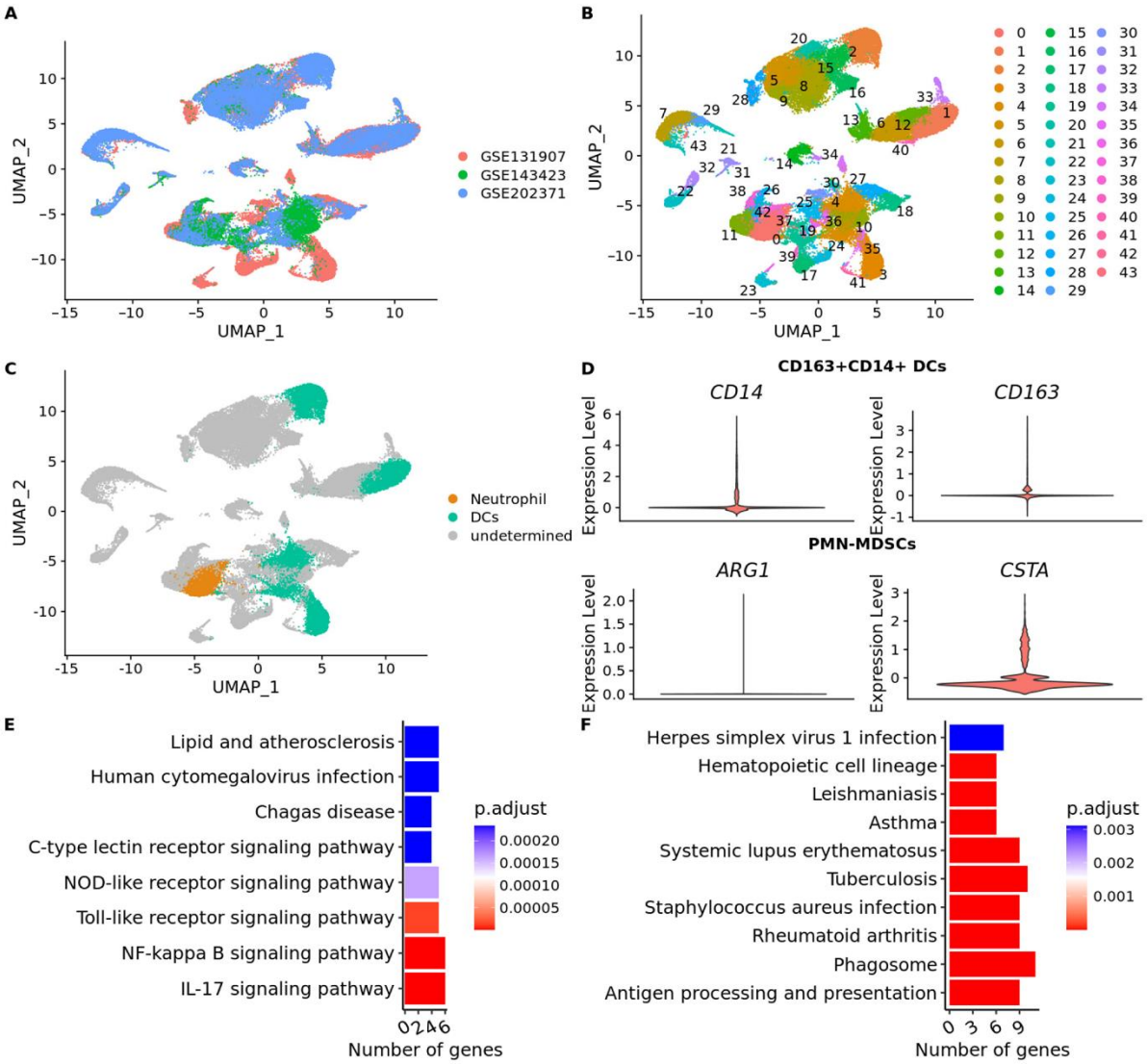

Figure 9. (**A**) The distribution of cells in each cluster according to the dataset. (**B**) 57,774 cells were divided into 43 separate clusters. (**C**) After annotation of cell type dendritic cells and neutrophils were found. (**D**) Violin plots of the expression of marker genes of neutrophil and dendritic cell subtypes. The violin plots to all the markers are shown in **Figure S4**. (**E**) Bar chart of enrichment analysis based on KEGG database for PMN-MDSCs. The *X*-axis shows the number of genes that enrich the pathway and the color of the bars represents the adjusted *p* value. (**F**) Bar chart of enrichment analysis based on KEGG database for CD163 + CD14+ dendritic cells. The *X*-axis shows the number of genes that enrich the pathway, and the color of the bars represents the adjusted *p* value.

4. Discussion

In the present study, we conducted a comprehensive search of public databases to obtain transcriptomic data of BM from patients diagnosed with adenocarcinoma as the primary tumor

(LUAD-BM). Differential expression analysis is the most commonly used method for identifying genes expressed aberrantly in the context of interest. These differentially expressed genes are then explored through functional annotation analyses and enrichment of specific deregulated pathways. Despite the clear convenience of the approach, it is limited by a high level of noise in gene expression data, difficulty in the reproducibility of results, and individual differences due to factors such as age, sex, genotype, and disease stage. Additionally, different treatment stages and variations in cohort and experimental methods can also result in disparities between studies. Therefore, combining multiple studies represented a powerful strategy to address these issues and extract relevant information from various datasets. The variability in transcriptome analysis technologies (RNA-seq and arrays) expands transcriptional profiles, increasing the number of possibilities for identifying key molecular pathways associated with BM in lung adenocarcinoma.

As can be observed in **Table 1**, most studies have a limited number of samples, and therefore, a comprehensive and integrative analysis of these data can reveal new molecular components that are not identified when these studies are analyzed individually. We recently comprehensively reviewed the transcriptomic changes that are associated with the development of LUAD-BM, including alterations in gene expression in both coding and non-coding RNAs [11]. Here, by combining multiple datasets, we have systematically identified consistently altered expression levels of 102 genes across a larger number of BM from LUAD. Remarkably, the majority of differentially expressed transcripts showed decreased expression in BM. Although our meta-analysis identified mainly protein-coding genes, noncoding RNAs, such as long non-coding RNAs (lncRNA) and circular RNAs (circRNA), represent an unexplored resource for identifying new contributors to LUAD-BM [11].

Results from KEGG pathway analysis indicated that these genes were enriched in pathways involving cell adhesion molecules, chemokine signaling, cytokine–cytokine receptor interaction, and differentiation pathways of Th1, Th2, and Th17 cells (**Table S8**). It is important to note that the most significantly altered immune-related pathway found was the cytokine–cytokine receptor interaction, which included 11/102 differentially expressed genes associated with BM from LUAD (*IL2RB*, *CCL5*, *CD27*, *CXCL13*, *CXCL9*, *CCL14*, *CXCL10*, *CCL18*, *CCL19*, *CCR7*, and *LTB*). Cytokine–cytokine receptor interactions can regulate immune responses by activating or inhibiting immune cells, including T cells, B cells, and natural killer (NK) cells [77]. These data suggest tissue specificity in the expression of some genes in BM and the

regulation of pathways mainly related to the immune system. Similar findings were reported by Tsakonas et al., who identified a pattern of decreased gene expression in BM of NSCLC-related genes primarily involved in immune response, immune cell activation, and cytokine and chemokine receptors [78]. Previous studies have demonstrated that inflammatory chemokines and their receptors regulate tumor cell migration and participate in tumor growth, metastasis, angiogenesis, and invasion through the interaction between mesenchymal cells and neoplastic cells [79,80].

To explore the specific characteristics of LUAD-BM, we analyzed the 102 genes related to BM in the specific context of the disease using the DO database and their cancer-specific relationship using the Network of Cancer Genes database. Our results showed no significant associations with specific cancers. These data suggest that LUAD-BM are distinct entities compared to the primary tumor, as reported in previous studies [16,20,81].

Additionally, protein–protein interactions among the DEGs were predicted. The interaction with the highest combined score was between the CD3D and CD247 proteins (combined score = 0.999). Both proteins are part of the TCR-CD3 complex present on the surface of T lymphocytes, which plays an essential role in the adaptive immune response. When antigen-presenting cells (APCs) activate the T-cell receptor (TCR), signals mediated by the TCR are transmitted through the cell membrane by the CD3, CD3D, CD3E, CD3G, and CD3Z chains [82]. In addition to its signaling role in T-cell activation, CD3D plays an essential role in thymocyte differentiation by participating in the assembly and proper surface expression of the intracellular TCR-CD3 complex. In the absence of a functional TCR-CD3 complex, thymocytes are unable to differentiate properly. CD3D also interacts with CD4 and CD8 and thus serves to establish a functional link between the TCR and the CD4 and CD8 co-receptors, which is necessary for the activation and positive selection of CD4 or CD8 T cells [83]. The TCR-CD3 complex represents a promising avenue for immunotherapy in metastatic brain cancer. The potential benefits of TCR-CD3-based interventions include potentiation of antigen recognition, immune activation, and immunosuppression reversal (for example, by providing T-cell-activating stimuli). There are several preclinical and clinical uses of CD3 modulators that may benefit patients suffering from brain metastasis [84].

After constructing the PPI network, we built a co-expression network in which we identified the top 20 elements of the network, also known as ‘hub’ genes. Of the 20 ‘hub’ genes, the CD69 gene had the highest degree (score = 396.192). The protein encoded by this gene is

involved in lymphocyte proliferation and functions as a signal transduction receptor in lymphocytes, NK cells, and platelets. It also regulates the differentiation of regulatory T cells (Tregs) as well as the secretion of IFN-γ, IL-17, and IL-22 [85]. These results support the hypothesis that the immune system plays a significant role in the development of LUAD-BM and suggests that targeting the immune system may be a promising approach for the treatment and management of LUAD-BM.

Previous studies have provided compelling evidence for the involvement of the immune system in the development of BM as reviewed by Leibold et al. [86]. It is well-established that the immune system plays a crucial role in regulating various stages of cancer progression, including the development and dissemination of metastatic tumors [87,88]. In the context of BM, immune cells and their interactions with cancer cells and the tumor microenvironment have been shown to have significant implications for progression [89]. Cytokines, chemokines, and growth factors play critical roles in the complex interplay between cancer cells and their surrounding environment during the development and progression of BM [90]. Recent studies have focused on identifying immunological characteristics specific to BM from NSCLC. Kudo et al. conducted a comparative immune gene profiling analysis and demonstrated elevated infiltration of M2 macrophages in BM compared to paired NSCLC samples [91]. Zhang et al. observed increased expression of CD163 M2 macrophages in the tumor brain microenvironment, which was correlated with a significant promotion of neo-angiogenesis [92]. Furthermore, Berghoff et al. found notable differences in the infiltration patterns of microglia and M2 macrophages between BM originating from NSCLC and melanoma [93]. Song et al. examined the expression of 770 genes related to the immune system across 28 different tissues, including primary tumors and BM of NSCLC. Utilizing the NanoString, Seattle, Washington, United States, nCounter PanCancer Immune Profiling Panel, they discovered that BM from EGFR-mutated adenocarcinoma exhibited increased activation of various immune-related pathways when compared to EGFR-wild-type adenocarcinoma. However, these same pathways were not observed in the primary tumors [94]. Additionally, the study discovered that the majority of immune cell subsets were reduced in BM in comparison to primary tumors. The reduction in immune cell subsets suggests the existence of possible immunosuppressive mechanisms within the environment of BM [94]. Recently, Najjary et al. used a combined approach based on NanoString's nCounter, immunohistochemistry, and the GeoMx™ Digital Spatial Profiler (DSP) to demonstrate a more extensive infiltration of immune cells in BM from lung adenocarcinoma compared to BM from

breast cancer [95]. Furthermore, the authors confirmed the higher protein expression of immune-related targets in BM-LUAD (CD14, CD163, GZMA, BCL-6, BAD, BCLXL, 4-1BB, VISTA, and IDO1). Interestingly, the gene *GZMA* was identified as a 'hub' gene in the present study.

GZMA, a member of the serine protease family, is primarily found in the cytolytic granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. It plays a significant role in cell-mediated cytotoxicity, which is a crucial immune response against tumor cells [96,97,98]. Previous studies have highlighted the importance of GZMA as a key effector molecule in regulatory T-cell function within the context of cancer [99,100]. Moreover, GZMA has been identified as a vital factor in inhibiting tumor growth, promoting apoptosis, and stimulating antigen-specific cytotoxic CD8+ T-lymphocytes [101]. Notably, Zhou et al. demonstrated that GZMA, derived from cytotoxic lymphocytes, specifically activates the gasdermin-B protein, contributing to the elimination of target cells [102].

Recent investigations by Huo et al. have revealed lower *GZMA* expression in breast cancer tissue compared to normal tissue. Additionally, a correlation has been observed between *GZMA* and T-cell checkpoints, including PD-1, PD-L1, and CTLA-4, in breast cancer [103]. Furthermore, quantitative immunofluorescence analysis has demonstrated a positive association between *GZMA* expression and the presence of dendritic cells and CD8+ T cells infiltrating breast cancer tissue. These findings suggest a positive association between *GZMA* expression and enhanced infiltration of dendritic and CD8+ T cells in breast cancer. In our study, we observed a significant positive correlation ($p < 0.05$) between *GZMA* expression and the infiltration of CD8 T cells and dendritic cells. Additionally, we found a positive correlation ($p < 0.05$) between CD8 T-cell infiltration and monocytes, which represent another subset of myeloid cells. Interestingly, *GZMA* expression was found to be associated with immune stimulators such as CD48 and CD27 [103]. These genes were identified as downregulated hub genes in our study. Based on these observations, we hypothesize that the downregulation of *GZMA* in LUAD-BM may play a crucial role in modulating immune cell infiltration and contribute to the establishment of a suppressive immune microenvironment.

Furthermore, our study revealed that the proportions of resting memory CD4 T cells comprised the largest cellular fraction of the total immune cells, accounting for 17.4% of the total immune cells in BM and 15.24% in primary tumors. Compared to primary tumors, the

proportions of resting dendritic cells and neutrophils showed statistical significance by the Wilcoxon-Mann-Whitney test ($p < 0.05$). Resting DCs were reduced in BM compared to primary tumors, while neutrophils showed an increased fraction. DCs are known for their essential role in activating the anti-tumor immune response through phagocytosis and the presentation of antigens from apoptotic tumor cells to CD4+ and CD8+ T cells. Normally, DCs are not found in the normal brain parenchyma but are present in vascular-rich compartments such as the choroid plexus and meninges [104]. In the context of pathological conditions such as cancer, DCs can migrate to the brain through afferent lymphatic vessels or endothelial venules [105].

Supporting our findings, Kim et al. also demonstrated the presence of DCs in LUAD-BM using scRNA-seq analysis [106]. Specifically, they identified CD163 + CD14+ DCs as the predominant subset in LUAD-BM [106]. Notably, CD163 + CD14+ DCs were found to be abundant in early- and advanced-stage lung cancer primary tissues but less abundant in metastatic lymph nodes and LUAD-BM [106]. DCs play a crucial role in the immune response by recognizing pathogens, coordinating both innate and adaptive immune responses, and secreting inflammatory mediators. DCs are unique in their ability to activate and direct naive T cells towards various effector cell types, such as Th1, Th2, Th17, and Tregs, depending on the specific cytokine and costimulatory signals they provide [107,108]. CD163 + CD14+ DC subset has been shown to possess a strong Th17 polarizing capacity, as evidenced by the pro-Th17 gene signature [109], which was consistent with our results (**Figure 9**E). Interestingly, our analysis revealed a lower fraction of DCs in BM compared to the primary tumor. This decrease in DC abundance within the BM microenvironment suggests the presence of an immunosuppressive environment that may have implications for the optimal presentation of tumor antigens in LUAD-BM. Furthermore, the absence of activated DCs in the BM microenvironment further supports the notion of an immunosuppressive setting. These findings highlight the possibility of sub-optimal tumor antigen presentation within LUAD-BM, potentially impairing the generation of effective anti-tumor immune responses. The immunosuppressive microenvironment observed in BM may contribute to the evasion of immune surveillance and facilitate tumor progression. However, further investigation is necessary to elucidate the underlying mechanisms responsible for the observed decrease in DCs abundance and the absence of activated DCs in the BM microenvironment.

Furthermore, our findings support the notion that polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) may play a role in creating an immunosuppressive

microenvironment. PMN-MDSCs have emerged as a distinct population of myeloid cells with immunosuppressive properties [110]. In the context of cancer, PMN-MDSCs have been implicated in the establishment of an immunosuppressive microenvironment that facilitates tumor growth and inhibits anti-tumor immune responses [110]. These cells have the ability to suppress the activity of various immune cells, including T cells, natural killer cells, and dendritic cells, thereby impairing the host's capacity to mount an effective immune response against cancer cells. Additionally, PMN-MDSCs contribute to tumor progression by promoting angiogenesis, tissue remodeling, and metastasis [110]. In line with our observations, Sun et al. have also identified the presence of PMN-MDSCs in gliomas and lung cancer brain metastases [111]. Notably, the authors demonstrated a high expression of L-selectin in PMN-MDSCs, which has been reported to regulate human neutrophil transendothelial migration [112].

Previous studies have shed light on the immune cell composition within primary brain tumors, with macrophages being identified as the predominant immune cell type, often constituting up to 30% of the tumor mass [113,114,115]. Wang et al. specifically demonstrated an increase in type-2 (M2) polarized macrophages in mesenchymal gliomas [116]. Furthermore, Liang et al. showed that neutrophils contribute to glioblastoma progression by supporting the expansion of the glioma stem cell pool through a S100 protein-dependent mechanism [117]. S100 proteins have been associated with the dissemination of breast cancer and are upregulated in the premetastatic brain, promoting neutrophil recruitment and subsequent metastatic seeding [118,119]. Interestingly, our observations in LUAD-BM align with these findings, suggesting potential common immune microenvironment features between BM and primary brain tumors. As a result, there may be opportunities for immunotherapeutic strategies, such as targeting tumor-associated neutrophils (TANs), that could be applicable to both LUAD-BM and primary brain tumors. Moreover, significant progress in the development of efficient delivery methods for immunotherapy, including nanocell-based drug delivery systems and drug repurposing [120], reinforces the potential of utilizing immunotherapy in treating LUAD-BM as well as primary brain tumors.

Overall, our study provides valuable insights into the complex immune microenvironment of LUAD-BM. The identified genes and pathways offer potential targets for therapeutic interventions aimed at overcoming immunosuppression and improving patient outcomes. However, further investigations are needed to elucidate the underlying mechanisms responsible for the observed changes in immune cell subsets and the immunosuppressive

microenvironment in BM. Additionally, exploring the role of PMN-MDSCs could provide further insights into their contribution to the immunosuppressive tumor microenvironment.

5. Conclusions

In conclusion, we identified 102 genes with altered expression levels related to LUAD-BM, with most showing decreased expression in BM. Pathway analysis revealed enrichment in genes involved in cell adhesion molecules, chemokine signaling, cytokine–cytokine receptor interaction, and differentiation pathways of Th1, Th2, and Th17 cells. Our analysis also identified key 'hub' genes, including *CD69* and *GZMA*, which are involved in lymphocyte proliferation, immune cell activation, and cytokine regulation. We found that the downregulation of *GZMA* in LUAD-BM may contribute to the establishment of a suppressive immune microenvironment. Furthermore, we observed alterations in immune cell populations in the brain metastatic microenvironment, including a decrease in dendritic cells and an increase in neutrophils, indicating the presence of an immunosuppressive environment within LUAD-BM.

Our findings highlight the importance of the immune system in the development and progression of LUAD-BM. Targeting the immune system may hold promise as a therapeutic approach for the treatment and management of BM in patients with adenocarcinoma. Further investigation is warranted to elucidate the underlying mechanisms driving the immune system dysregulation in LUAD-BM and to explore potential immunotherapeutic strategies to improve patient outcomes.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15184526/s1. Figure S1: Count distribution in RNA-Seq data; Figure S2: Distribution of counts in microarray data; Figure S3: ScRNA-seq quality control plots; Figure S4: Quality assurance for single-cell RNA sequencing data; Figure S5:Violin plots showing the expression levels of cell type marker genes; Table S1: Clinical information available for the studies included in the meta-analysis; Table S2: Number of reads

before and after quality control with SeqyClean; Table S3: Alignment statistics with STAR. Aggregated results with MultiQC; Table S4: Differentially expressed genes in BM in comparison to the primary tumor using RNA-Seq data; Table S5: Differentially expressed genes in BM in comparison to the primary tumor using microarray data; Table S6: Overlapping between RNA-Seq and microarray; Table S7: Genes with the same expression direction between the two sequencing platforms; Table S8: Enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway; Table S9: Gene Ontology (GO) enrichment analysis; Table S10: Disease Ontology (DO) enrichment analysis; Table S11: Enrichment analysis based on the Network of Cancer Genes database; Table S12: Protein–Protein interaction network (PPI); Table S13: 'Hub' genes and the score grades; Table S14: Gene Ontology (GO) enrichment analysis—'hub' genes; Table S15: Enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG)—'hub' genes; Table S16: The association between 22 immune cell subtypes in BM by Spearman's correlation analysis; Table S17: The association between 22 immune cell subtypes in the primary tumor by Spearman's correlation analysis; Table S18: Top 30 markers per cluster; Table S19: KEGG pathway enriched in neutrophils; Table S20: KEGG pathway enriched in dendritic cells.

Author Contributions

P.P.R. and V.G.P.S. initiated the project. V.G.P.S. designed, researched, analyzed, and wrote the topics covered in the article. V.G.P.S. designed the figures. V.G.P.S., A.F., N.T., G.L.S., R.F.C., L.A.J.M., P.P.R. and W.L.L. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The datasets that support the findings of this study are available in the European Genome-phenome Archive (EGA) under accession numbers EGAS00001004078 and EGAS00001004006; Sequence Read Archive (SRA) data under accession number PRJNA510710; Gene Expression Omnibus (GEO) under accession numbers GSE141685, GSE60645, GSE131907, GSE143423, and GSE202371; and the functional genomics data collection (ArrayExpress) under accession number E-MTAB-8659.

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Conflicts of Interest

The authors declare no conflict of interest.

References

The references used in this chapter will be presented at the end of thesis.

4.2 Capítulo 3

Comprehensive Analysis of Lung Adenocarcinoma and Brain Metastasis through Integrated Single-Cell Transcriptomics

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Abstract

Lung adenocarcinoma (LUAD) is a highly prevalent and lethal form of lung cancer, comprising approximately half of all cases. It is often diagnosed at advanced stages with brain metastasis (BM), resulting in high mortality rates. Current BM management involves complex interventions, and conventional therapies that offer limited survival benefits with neurotoxic side effects. The tumor microenvironment (TME) is a complex system where cancer cells interact with various elements, significantly influencing tumor behavior. Immunotherapies, particularly immune checkpoint inhibitors, target the TME for cancer treatment. Despite their effectiveness, it is crucial to understand metastatic lung cancer and the TME's specific characteristics, including cell-cell communication mechanisms in order to refine treatments. This study integrates multiple single-cell transcriptomic sequencing datasets, including LUAD at different stages and BM samples to elucidate the main cellular and molecular mechanisms that govern the development and progression of BM. Our results showed downregulated genes within the human leukocyte antigen complex in neutrophils, macrophages, and dendritic cells from BM, suggesting their role in disease progression. Stage-specific sub-clustering analyses

identified distinct immune cell profiles across tumor stages, emphasizing the dynamic nature of immune responses in primary tumors and BM. Our study sheds light into the molecular aspects of BM-LUAD, providing essential knowledge for future research. Emphasizing the potential of immunotherapies and personalized approaches, our findings may contribute to the management of this challenging disease, with the ultimate goal of improving patient outcomes.

Keywords: Lung cancer; NSCLC; Tumor microenvironment; Brain metastasis

1. Introduction

Lung cancer is the leading cause of cancer-related deaths [1]. Lung adenocarcinoma (LUAD) is the most common histological subtype of lung cancer, accounting for roughly 40-50% of all lung cancer cases [2,3]. LUAD is commonly detected at advanced stages with regional and/or distant metastasis that can affect the brain, leading to the currently observed high mortality rates. Compared to other types of lung cancer, LUAD has a higher tendency to metastasize to the brain. The process of brain metastasis (BM) involves several steps, including migrating cancer cells to the brain, establishing a metastatic focus, and interacting with the brain's microenvironment [4]. Managing BM is one of the most difficult clinical challenges requiring multidisciplinary approaches, mainly comprising local interventions such as surgery, radiotherapy, and palliative care including treatment with corticosteroids [4]. However, conventional therapies offer only marginal survival benefits and are often associated with high morbidity rates due to the neurotoxic effects, which may lead to cognitive impairment and other neurological complications [5–7].

The tumor microenvironment (TME) is a complex ecosystem where cancer cells interact with immune cells, stromal cells, blood vessels, and extracellular matrix, and where these interactions can significantly impact tumor behavior and cancer progression [8]. In the context of immunotherapies, targeting the TME is an attractive strategy for treating cancer [9]; for example, immune checkpoint inhibitors (ICIs) are antibody-based therapies targeting immune cells in the TME [10]. ICIs have been approved as early as 2011 to treat unresectable advanced melanoma after conventional therapy [11], and as of September 2022, the US Food and Drug Administration (FDA) has approved nine drugs targeting four immune checkpoints, including:

cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed cell death-1 (PD-1), programmed death ligand-1 (PD-L1), and lymphocyte activation gene-3 (LAG-3).

Over the past decade, immunotherapy with ICIs has made a significant breakthrough in advanced lung cancer treatment, offering these patients substantial improvements in survival and quality of life [12]. In the context of BM treatment, emerging data suggests that ICIs exhibit promising activity and safety in non-small cell lung cancer (NSCLC) patients with BM [13]. However, more studies are needed to understand the molecular features that characterize metastatic lung cancer and the complex microenvironment supporting its progression. A comprehensive understanding of these factors is crucial for refining treatment strategies and improving patient outcomes. Moreover, understanding the heterogeneity of the TME allows for the identification of potential biomarkers that may predict response or resistance to immunotherapies. Tailoring treatment strategies based on the unique characteristics of the TME may improve patient outcomes and expand the success of immunotherapies in BM from LUAD (BM-LUAD).

Studies utilizing single-cell transcriptomic sequencing (scRNA-Seq) have successfully unveiled pivotal molecular insights into individual metastatic cells in lung cancer and BM, where these investigations have provided valuable findings concerning the molecular and cellular reprogramming of metastatic lung adenocarcinoma, the tumor heterogeneity of lung cancer, and the diversity present in the tumor microenvironment of BM [14–17]. Nonetheless, one of the main challenges in studying molecular changes associated with BM is the limited number of samples for analysis, as BM are usually not resectable, leaving only the primary tumors accessible.

In order to address this challenge, we integrated multiple scRNA-seq datasets derived from BM-LUAD and primary tumors across various stages (I, II, III, and IV), outlined in **Figure 1**. Our primary focus was the immune cell composition and the expression profiles and functions of cell type-specific genes. Additionally, we explored cellular interactions within the TME by inferring cell-cell communication mechanisms, utilizing a database of ligands and receptors. Overall, our study provides additional insights for further research on the TME-immune ecosystem and immunotherapy for BM-LUAD.

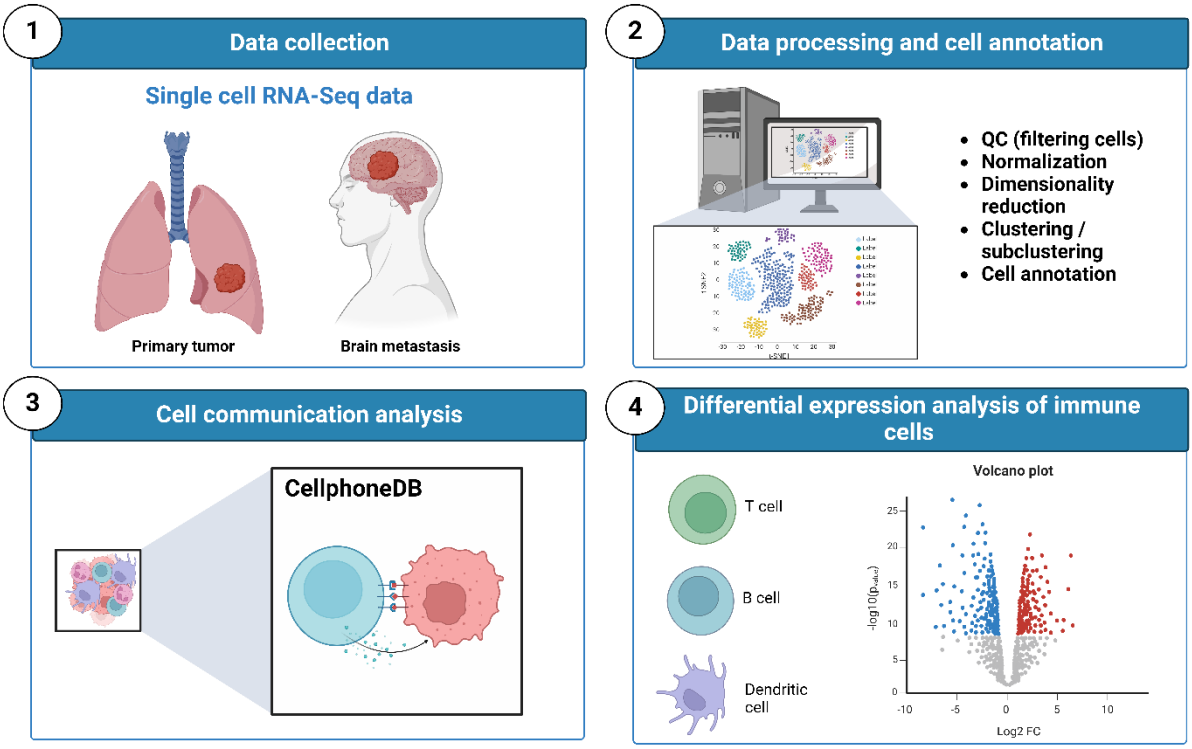

Figure 1. Study Design: Three independent datasets were collected from GEO (see **Table 1**), and subsequently underwent pre-processing, quality control, normalization, and clustering using the Seurat package. Dataset integration was executed through an anchor-based approach, with annotations derived from the CellMarker2.0 database. Subsequent cell communication analysis unveiled interactions between cells. Following this, differentially expressed genes between immune cell types were identified using the FindMarkers function from the Seurat package.

2. Results

2.1. Processing of single-cell RNA sequencing data

We obtained expression data from three scRNA-Seq datasets available on the Gene Expression Omnibus (GEO) database. These datasets included 23 samples from BM and 18 samples from primary tumors, covering stages I, II, III, and IV disease **(Table 1)**. The total number of cells examined was 148,905, and from these, we evaluated the number of unique genes detected in each cell to identify low-quality cells, empty droplets, or cell doublets/multiplets (nFeature_RNA) **(Figure 2A)**. Additionally, we assessed the total number of molecules detected within a cell (nCount_RNA) and mitochondrial gene expression (percent_mt) **(Figure 2A)**. Elevated expression levels of mitochondrial genes could indicate

poor sample quality, suggesting a high occurrence of cell apoptosis or lysis and low cell activity.

A total of 136,946 cells (GSE131907, *n*=86,274; GSE143423, *n*=12,196; and GSE202371, *n*=38,476) were identified after quality control. Only cells meeting the quality criteria of nFeature_RNA > 200 and < 9,000, and percent.mt < 20 were retained for downstream analysis **(Figure 2B)**. We employed a FeatureScatter plot to visualize feature-feature relationships before the quality control **(Figure 2C)** and after the quality control **(Figure 2D)**. Figure 2D displays a positive correlation of 0.90 between sequencing depth and the number of detected genes. This positive correlation is desirable as it implies a higher sequencing depth result in a more comprehensive and accurate representation of the cellular transcriptome. After conducting quality control filtering, we detected a positive correlation of 0.11 between sequencing depth and mitochondrial gene content. This means that as sequencing depth increases, mitochondrial gene content also tends to increase. However, the correlation is weak, which suggests that other factors may also influence mitochondrial gene content.

Table 1. Description of single-cell transcriptomic data used in this study.

Database	Access	Platform	Stage	No. of Samples	No. of cells*	Ref
Brain metastasis						
GEO	GSE131907	Illumina HiSeq 2500	IV	10	29057	[14]
GEO	GSE202371	Illumina NovaSeq 6000	IV	10	38476	[14]
GEO	GSE143423	HiSeq X Ten	IV	3	12196	NA
Primary tumor						
GEO	GSE131907	Illumina HiSeq 2500	I	8	31025	[14]
GEO	GSE131907	Illumina HiSeq 2500	II	2	3840	[14]
GEO	GSE131907	Illumina HiSeq 2500	III	4	10282	[14]
GEO	GSE131907	Illumina HiSeq 2500	IV	4	12070	[14]

* Number of cells after quality control. NA: Not available. No.: Number.

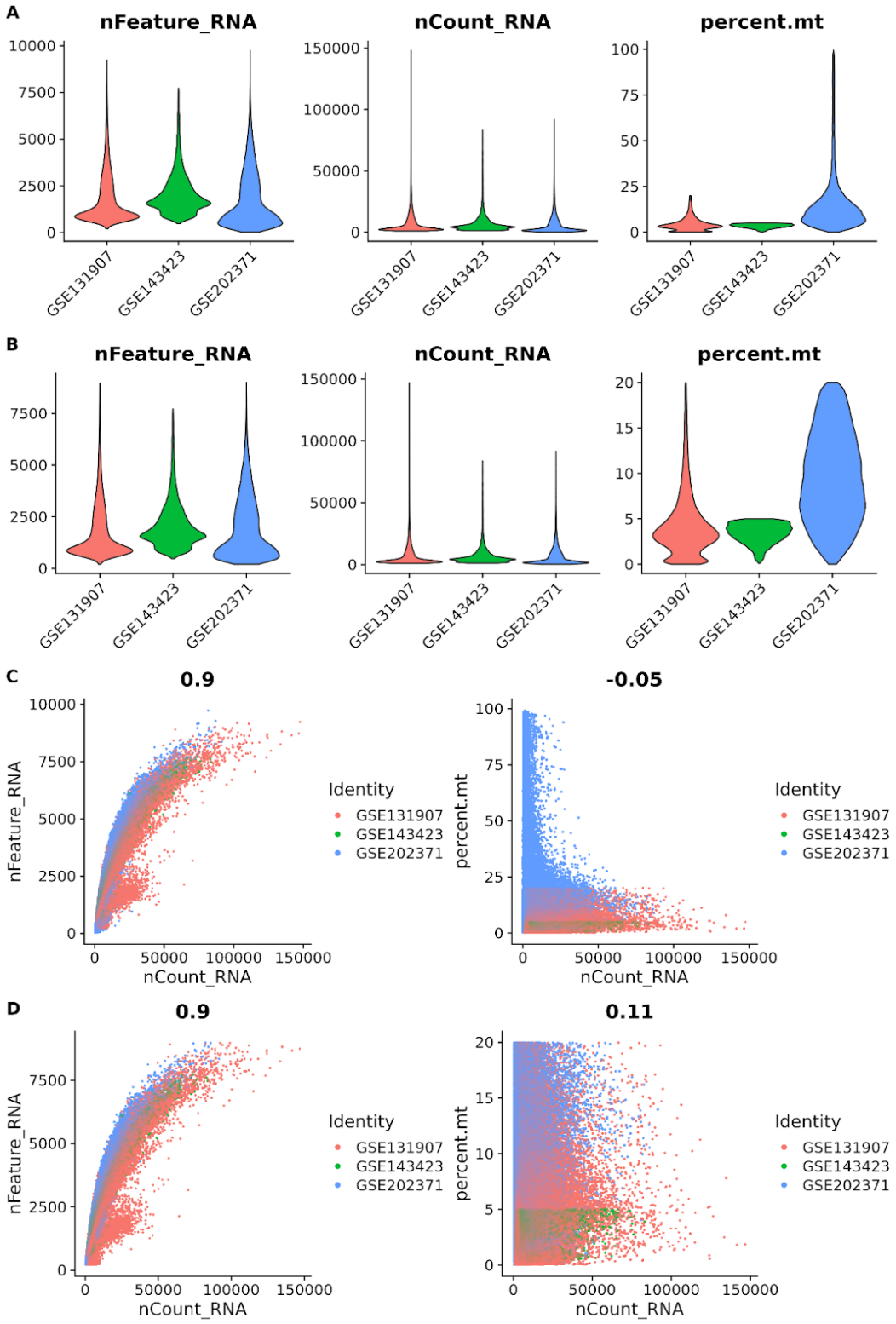

Figure 2. Quality of the final dataset. Violin plots depict QC metrics of cells in the final dataset, illustrating the number of unique genes detected in each cell (nFeature_RNA), the total number of molecules detected within a cell (nCount_RNA), and mitochondrial gene expression (percent_mt), categorized by study. **(2A)** displays Violin plots before quality control, while **(2B)** shows them after quality control. Additionally, FeatureScatter plots visualize feature-feature relationships before **(2C)** and after **(2D)** quality control analysis.

2.2. Principal component analysis and batch effect correction

After the quality control analysis, datasets were integrated using identified anchors, and downstream analysis was conducted. Principal Component Analysis (PCA) was utilized to select a set of linearly independent variables known as principal components (PCs). The Elbowplot function helped determine the number of principal components, showcasing the contribution of each PC ranked according to the percentage of variance. The elbow plot revealed an 'inflection point' around PC15, signifying that the first fifteen principal components captured most of the true signals. Consequently, we selected these 15 principal components for subsequent analysis ($p < 0.05$). PCA was then performed on the expression data for dimensionality reduction analysis **(Figure 3)**. The integration of the three datasets of BM samples after normalization and batch effect correction is presented in **Figure 3A**.

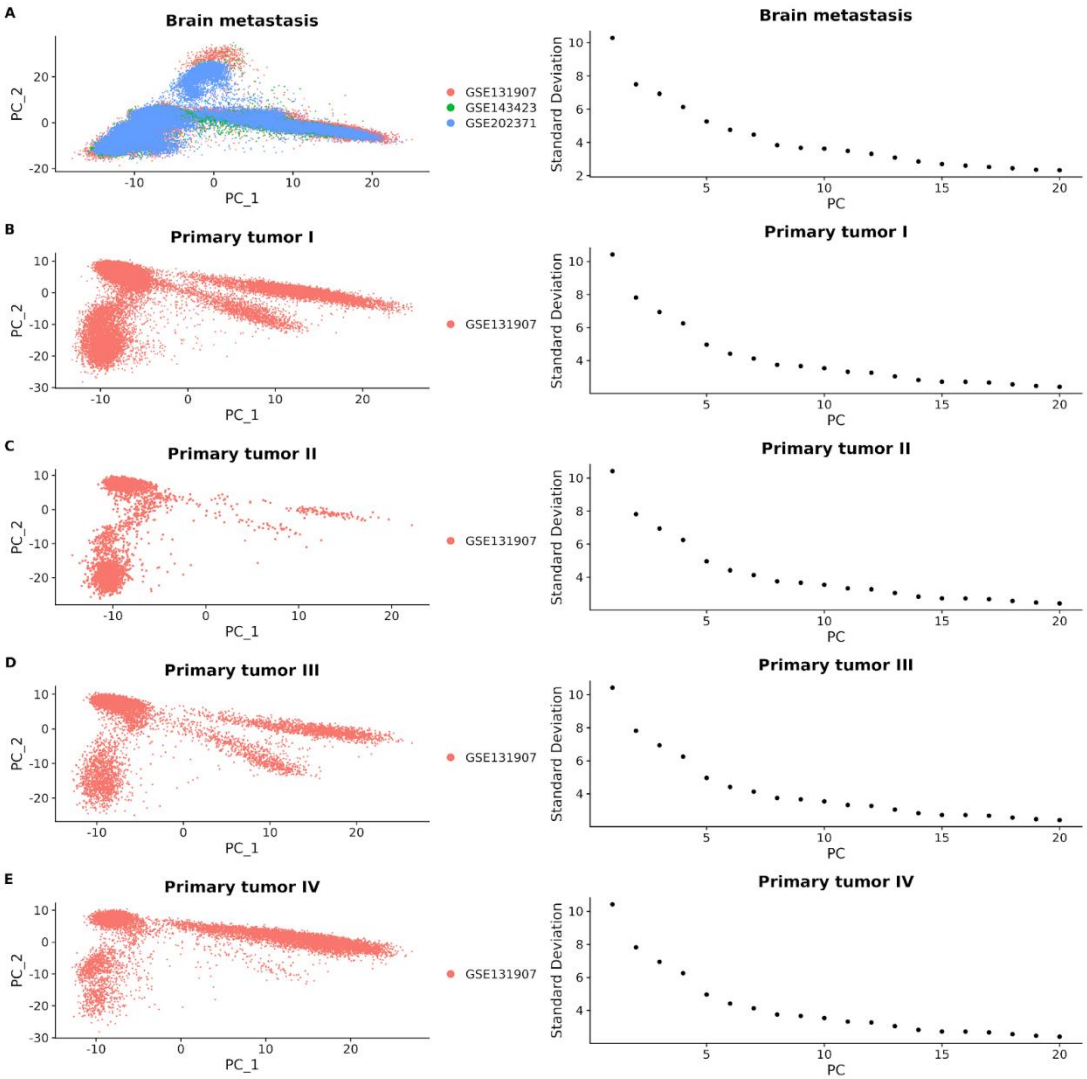

Figure 3. DimPlots and elbow plots display PCA results for **(A)** brain metastasis, **(B)** primary tumor stage I, **(C)** primary tumor stage II, **(D)** primary tumor stage III, and **(E)** primary tumor stage IV.

2.3. Primary Tumors and Brain Metastases Exhibit Different Immune and Stromal Infiltration Patterns.

After data processing and quality control, we successfully cataloged 136,946 cells, each assigned to distinct cell types through annotation based on expressed marker genes. Our classification comprised five non-immune cell types, including endothelial cells, fibroblasts, epithelial cells (EPCs), astrocytes, and oligodendrocytes (the latter two only found in BM), alongside eight immune cell types: macrophages, dendritic cells (DCs), neutrophils, mast cells, T cells, NK cells, B cells, and microglia (present exclusively in BM) **(Figure 4)**.

We applied the Uniform Manifold Approximation and Projection (UMAP) method for sub-clustering and classified the cells from BM samples into 54 separate clusters **(Figure 4A)**, primary tumor stage I (TI) in 23 separate clusters **(Figure 4B)**, primary tumor stage II (TII) into 19 clusters **(Figure 4C)**, primary tumor stage III (TIII) into 20 clusters **(Figure 4D)** and finally primary tumor stage IV (TIV) into 26 clusters **(Figure 4E)**. Then we annotated the clusters as described in the methodology.

In BM samples we identified immune cells including macrophages (*n*=2,734), DCs (*n*=4,447), neutrophils (*n*=5,910), mast cells (*n*=732), T cells (*n*=13,818), NK cells (*n*=1,861), B cells (*n*=873), and microglia (*n*=14,813); and non-immune cells which included endothelial cells (*n*=7,267), fibroblasts (*n*=517), EPCs (*n*=8,698), astrocytes (*n*=4,211) and oligodendrocytes (*n*=911) **(Figure 4A)**. Functional enrichment analysis suggested that the COX reactions pathway is significantly upregulated while the ATP-sensitive potassium channels pathway is downregulated in macrophages, microglia, DCs, Mast cells, and EPCs **(Supplementary Figure S1)**. Furthermore, all of these cells exhibit upregulation of Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation pathway **(Supplementary Figure S2)**. **Table 2** shows the number of cells per tumor stage in primary tumor samples. It is important to note that TIV showed no presence of mast cells and in TIII there were no neutrophils. Also, fibroblasts were too few to detect in stages I and II **(Figures 4B-E)**.

Table 2. Number of cells per tumor stage in primary tumor samples.

	Stage I	**Stage II**	**Stage III**	**Stage IV**
Immune cells				
Macrophages	3,801	483	1,411	526
NK cells	3,439	517	292	732
Neutrophils	651	353		636
Dendritic Cells	1161	232	295	457
B cells	324	133	748	390
Mast cells	1480	222	235	
T cells	13125	1373	4865	2696
Non-immune cells				
Endothelial	456	100	174	153
Fibroblasts			418	228
Epithelial cells	2670	220	467	4970

Subsequently, we quantified the proportion of each cell type in both primary tumors and BM **(Figures 5A** and **B)**. Notably, immune cells exhibited higher proportions in both primary tumors and BM **(Figure 5C)**, constituting 87% for TI, 91% for TII, 88% for TIII, 51% for TIV, and 68% for BM. T cells emerged as the predominant immune cell type across all primary tumors, representing 48%, 38%, 55%, and 25% for stages I, II, III, and IV, respectively. Meanwhile, microglia (22%) stood out as the primary immune cell type in BM.

Moreover, TI is marked by a 14% presence of macrophages, accompanied by significant contributions from NK cells, EPCs, and DCs. In TII, NK cells take prominence at 14%, while macrophages, neutrophils, DCs, and mast cells collectively constitute 40%. TIII is characterized by 16% macrophages and 8% B cells, with EPCs, fibroblasts, DCs, and NK cells contributing a combined 20%. TIV is distinguished by EPCs as the major component at 46%, alongside NK cells, neutrophils, and macrophages constituting 19%. B cells, DCs, fibroblasts, and endothelial cells collectively account for 13% **(Supplementary Table S1)**.

Functional enrichment analysis indicates that most primary tumors downregulated the FGFR1c and Klotho ligand binding and activation pathway **(Supplementary Figures S3-S6)**.

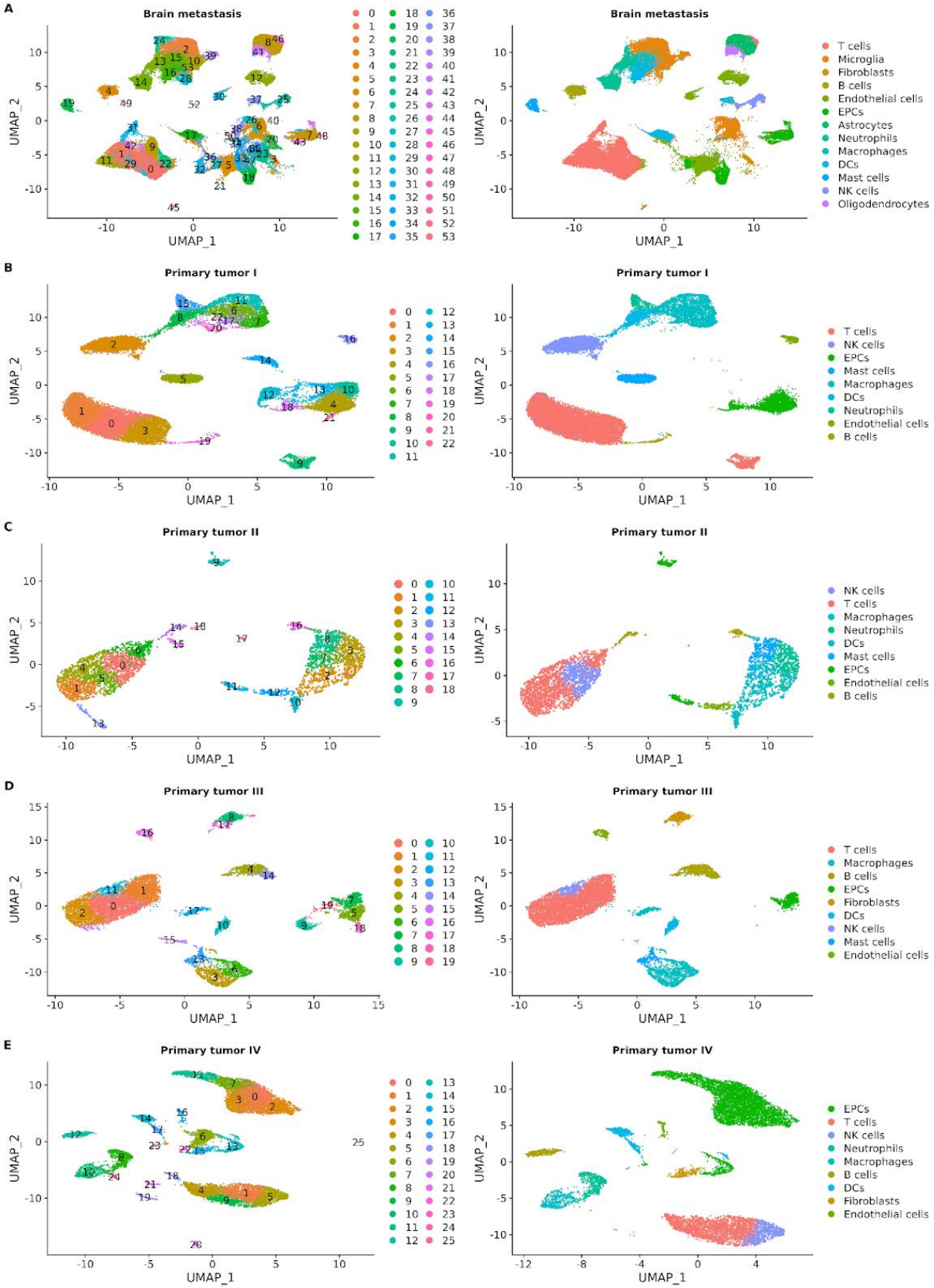

Figure 4. UMAP plots showing the main cell types annotated by known gene markers. **(A)** Brain metastasis. **(B)** Primary tumor stage I. **(C)** Primary tumor stage II. **(D)** Primary tumor stage III. **(E)** primary tumor stage IV. DCs: Dendritic cells. EPCs: Epithelial cells.

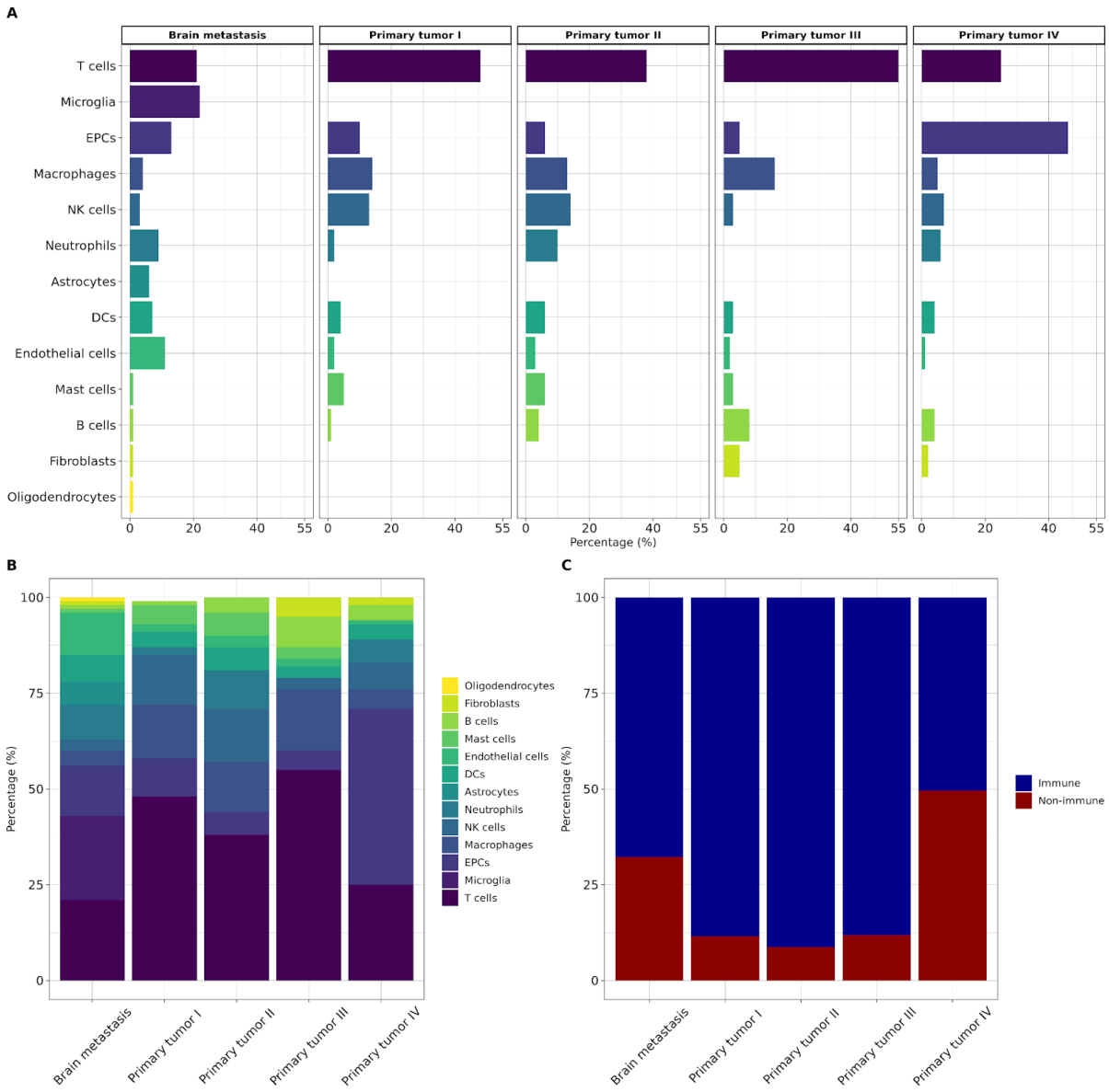

Figure 5. (A, B) The bar plot displays a comparison of the proportions of the main cell types between brain metastasis and primary tumors across four stages (I, II, III, and IV). **(C)** The bar plot shows the composition of immune and non-immune cells in both brain metastasis and primary tumors. DCs: Dendritic cells. EPCs: Epithelial cells.

2.4 Complex Intercellular Communication Networks in Brain Metastasis

Next, we identified ligand-receptor pairs and molecular interactions among the major cell types **(Figures 6A-E)**. Broadcast ligands, for which cognate receptors were detected, demonstrated extensive communication between immune and non-immune cells, underscoring their crucial roles in the communication between immune and non-immune cells within the TME in the development and progression of lung cancer.

Notably, BM exhibited a higher number of significant ($p < 0.05$) potential interactions compared to primary tumors **(Supplementary Tables S2-S6)**. Remarkably, in BM samples, endothelial cells exhibited a higher number of interactions with both microglia and oligodendrocytes (10 and 8, respectively) **(Figure 6A)**. Additionally, by using this integrative approach, we were able to identify that microglia primarily interact with oligodendrocytes and NK cells.

Among the most remarkable findings, a pivotal interaction was identified between *DLL4* and *NOTCH4*, demonstrating a significant ($p < 0.05$) association from endothelial cells to microglia and from microglia to oligodendrocytes. Another noteworthy interaction was found between *VEGFC* and *KDR*, revealing a significant association between microglia and oligodendrocytes. Additionally, the interaction between *LRFN4* and *PTPRD* showed a significant association from endothelial cells to oligodendrocytes **(Figure 6F)**.

Additional notable findings included *KISS1-KISS1R* interactions, associated both from microglia to endothelial cells and from oligodendrocytes to endothelial cells. Similarly, the interaction between *WNT4* and *FRZB* demonstrated a significant association between microglia and oligodendrocytes **(Figure 6F)**. WNT4 and FRZB are two proteins involved in the Wnt signaling pathway, which regulates key cellular events during the development of the brain and is involved in the genesis of glioblastoma [18].

Additionally, we observed distinct cell-interaction profiles in primary tumors, with fewer interactions between immune and non-immune cells in stages I, II, and III (as shown in **Figures 6B-D**). However, from Stage IV onwards, we noted an increase in interactions between the immune and non-immune cells, with macrophages exhibiting a higher number of interactions, mainly with neutrophils, DCs, endothelial cells, and EPCs. It is noteworthy that previous studies have demonstrated the involvement of macrophages in tumor growth, migration, and metastasis [19], which aligns with the results we obtained.

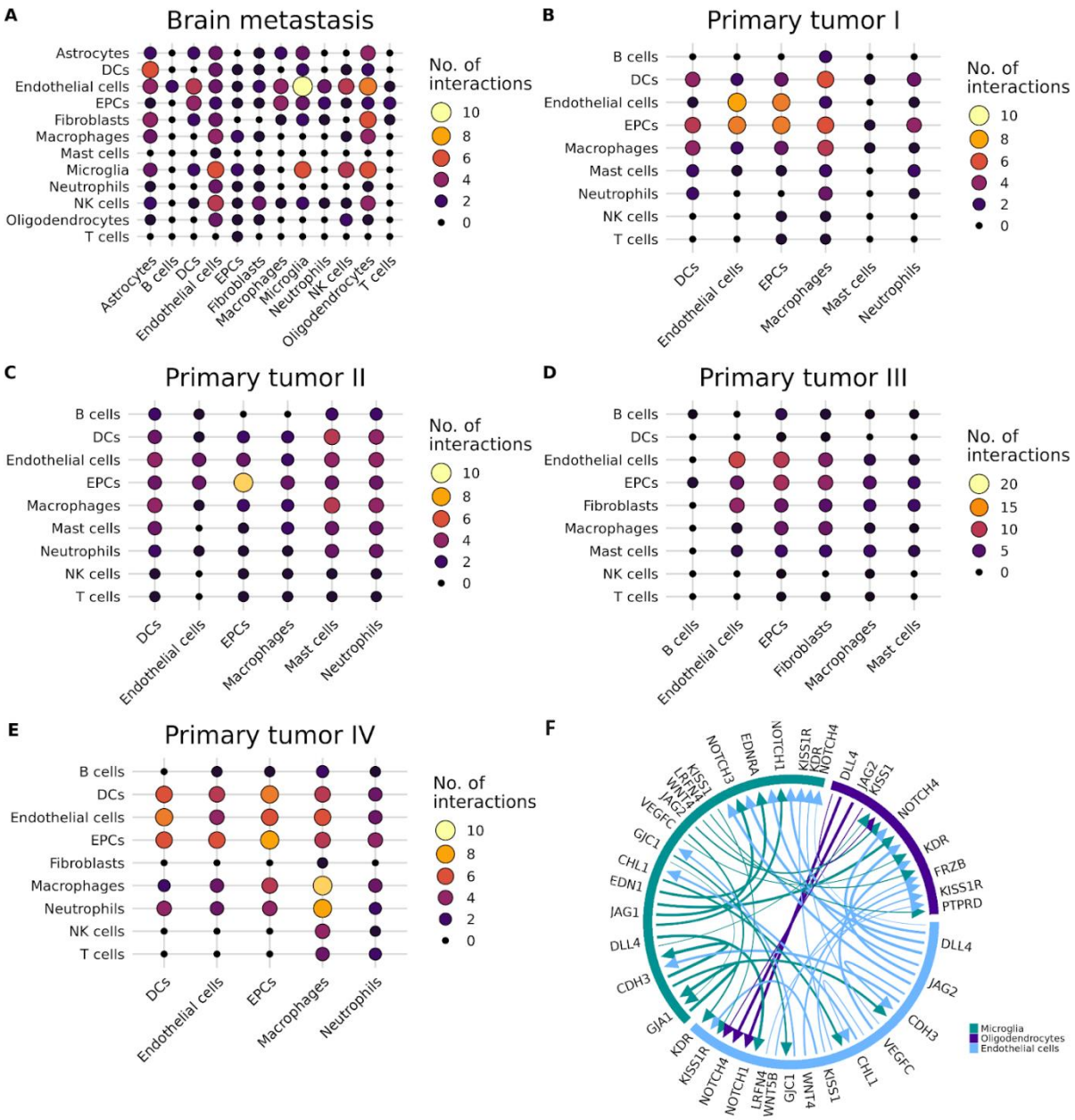

Figure 6. Inferred cell-cell signaling through ligand-receptor interaction analysis in CellPhoneDB. **(A, B, C, D, E)** Number of interactions between the cell types. Point size and colors represent the number of ligand-receptor interactions ($p < 0.05$). Node size represents the number of interactions. **(F)** Selected ligand–receptor interactions between microglia, oligodendrocytes, and endothelial cells in brain metastasis samples. The colors represent the cell types and the arrows represent the interaction between ligands and receptors. DCs: Dendritic cells. EPCs: Epithelial cells.

2.5. Immune Cells Reveal Potential Roles of the Human Leukocyte Antigen Complex (HLA) in Brain Metastasis Progression

Following the comprehensive characterization of cell types within each sample group, we proceeded to investigate the distinct expression patterns of genes, known as differentially

expressed genes (DEGs), within specific cell types across different stages of primary tumors (I, II, III, and IV) and BM. We specifically focused on the differential expression profiles of various immune cell populations present in both primary tumors and BM, such as T cells, macrophages, NK cells, neutrophils, DCs, mast cells, and B cells. The aim was to identify and elucidate differentially expressed genes for each specific cell type, thereby shedding light on the molecular intricacies underlying the immune landscape across different stages and tissue environments.

Distinct profiles of gene expression emerged across different comparisons (refer to **Supplementary Tables S7-S13**). Notably, we observed downregulation of several genes within the human leukocyte antigen (HLA) complex, including *HLA-DPB1* (log2FC = -1.25), *HLA-DPA1* (log2FC = -1.03), and *HLA-DQA2* (log2FC = -1.13) in neutrophils from BM when compared to neutrophils from primary tumor stage IV **(Figure 7A)**. Similarly, in macrophages from BM, we observed downregulation of several genes within the HLA complex (*HLA-DPB1*, *HLA-DRA*, *HLA-DPA1*, *HLA-DQB1*, *HLA-DQA1*, *HLA-DQA2*, and *HLA-DRB5*) when compared to macrophages from primary tumor stage IV **(Figure 7B)**. These genes enriched pathways such as Th1 and Th2 cell differentiation, and Th17 cell differentiation **(Figures 7D-F)**. Furthermore, subsequent gene ontology (GO) enrichment analysis demonstrated that these genes were associated with processes such as MHC class II protein complex assembly, peptide antigen assembly with MHC class II protein complex, and antigen processing and presentation **(Figures 7G-I)**. The results were similar for DCs from BM when compared to DCs from primary tumor stage IV **(Figures 7C, 7F, and 7I)**. The results of the GO enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) can be found in **Supplementary Tables S14** and **S15** respectively.

Additionally, T cells, one of the most abundant immune cells in all samples, exhibited significant gene dysregulation in the BM compared to primary tumors. Specifically, genes such as *PLP1*, *LUM*, and *IGFBP7* showed substantial alterations ($|\log2FC| > 2$) in T cells from BM when contrasted with T cells from primary tumor I. Conversely, T cells from BM displayed low expression of *MGP* (log2FC = -3.22) and elevated expression of *PLP1* and *HSPA1A* (log2FC = 2.33 and 2.03, respectively) in comparison with T cells from primary tumor II **(Supplementary Table S7)**.

Furthermore, when comparing T cells from BM with those from primary tumor IV, we observed significant alterations in the expression of genes such as *PLP1* (log2FC = 2.34),

HSPA1A (log2FC = 2.09), *HBB* (log2FC = -2.93), and *HBA2* (log2FC = -2.44). Moreover, genes *TFF3*, *SCGB3A1*, and *PLP1* consistently exhibited differential expression in T cells from BM when compared with T cells from primary tumors, suggesting a potential role for these genes in BM development **(Supplementary Table S7)**. Subsequent pathway enrichment analysis revealed that dysregulated genes in T cells from BM were associated with crucial lung cancer-related pathways, such as the PI3K-Akt signaling pathway, ECM-receptor interaction, and MAPK signaling pathway ($p < 0.0.5$) **(Supplementary Table S14)**.

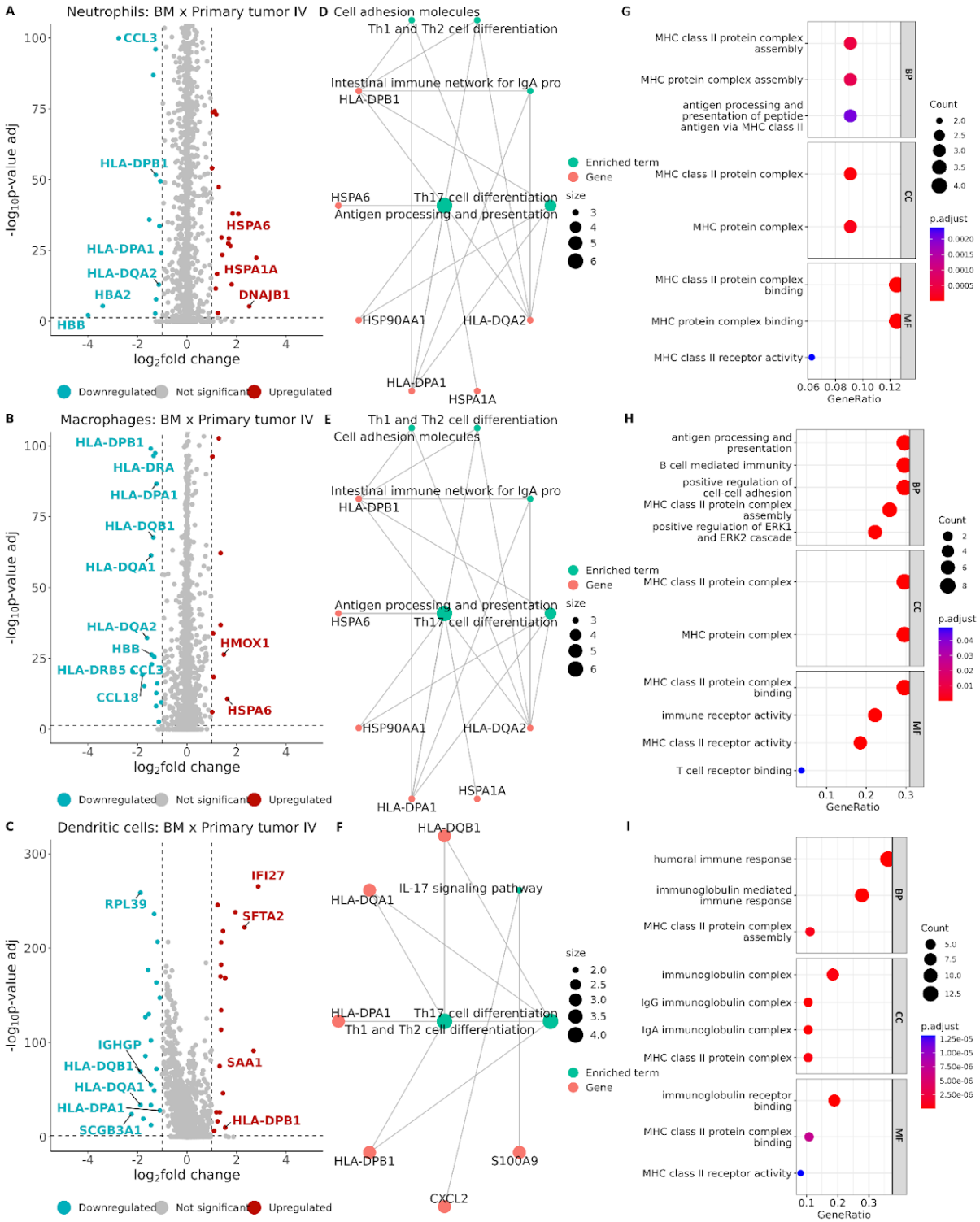

Figure 7. Differential expression of genes in specific cell types between brain metastasis and primary tumor stage IV. Volcano plots show -log10 adjusted *p*-value on the y-axis versus log2 fold change on the x-axis. **(A)** Neutrophils. **(B)** Macrophages. **(C)** Dendritic cells (DCs). The labels indicate genes that are part of the human leukocyte antigen (HLA) complex. Additionally, representative genes with highly significant fold changes are shown in the volcano plot. See Supplementary Tables S8, S10, and S11 for full lists of significantly changed genes in macrophages, neutrophils, and DCs, respectively. **(D, E, F)** Enrichment analysis interaction network from the Kyoto Encyclopedia of Genes and Genomes (KEGG), for neutrophils, macrophages, and DCs, respectively. The node size represents the number of genes according to each KEGG category, and the color of the nodes represents the enriched term (green) and gene (red), as shown by the legend. **(G, H, I)** Enrichment dot plot of the term Genetic Ontology

(GO). The graph displays the enriched ontologies associated with the genes presented in the volcano plot. Each of the instance terms BP = biological process, MF = molecular function, and CC = cellular component is represented ($p < 0.0.5$). The X-axis presents the number of genes that enrich the ontology term, and the point size is proportional to this number.

2.6. Stage-Specific Sub clustering Unveils Distinctive Profiles of Dendritic Cells in the Tumor Microenvironment

First, cells were clustered into major cell types as described previously. Subsequently, DCs, T cells, and B cell populations, which were the only three types of immune cells present in all the primary tumors and BM, were divided into subsets for normalization, dimensionality reduction, and further sub clustering stratification analysis. This approach allowed for the detection of heterogeneity within each cell type, considering their inherent diversity.

In a prior investigation, we conducted a detailed sub clustering analysis of DCs in BM samples, revealing the presence of CD163+CD14+ DCs [20]. Expanding our focus to primary tumor stages I, II, III, and IV, we investigated the diverse subtypes of DCs, as well as T cells and B cells, in both BM and primary tumors. Our reclassification of DCs into six subsets, including CD1c+ DCs (Langerhans cells, LCs), CD141+ DCs, CD207+CD1a+ LCs, plasmacytoid DCs (pDCs), CD163+CD14+ DCs, and activated DCs, uncovered significant heterogeneity.

In primary tumor stage I, we subclustered 1,161 DCs into seven distinct subclusters, identifying subcluster 1 as activated DCs (*n*=211 cells), subcluster 2 as CD163+CD14+ DCs (*n*=191 cells), and subcluster 4 as pDCs (*n*=145 cells) **(Figure 8A)**. Moving to primary tumor stage II, we subclustered 232 DCs into four subclusters, with subcluster 3 representing CD163+CD14+ DCs (*n*=48 cells) **(Figure 8B)**. For primary tumor stage III, our sub clustering of 295 DCs into four subclusters revealed cluster 3 as pDCs (*n*=34 cells) **(Figure 8C)**. Finally, in primary tumor stage IV, we subclustered 457 DCs into five subclusters, pinpointing subcluster 4 as activated DCs (*n*=43) **(Figure 8D)**.

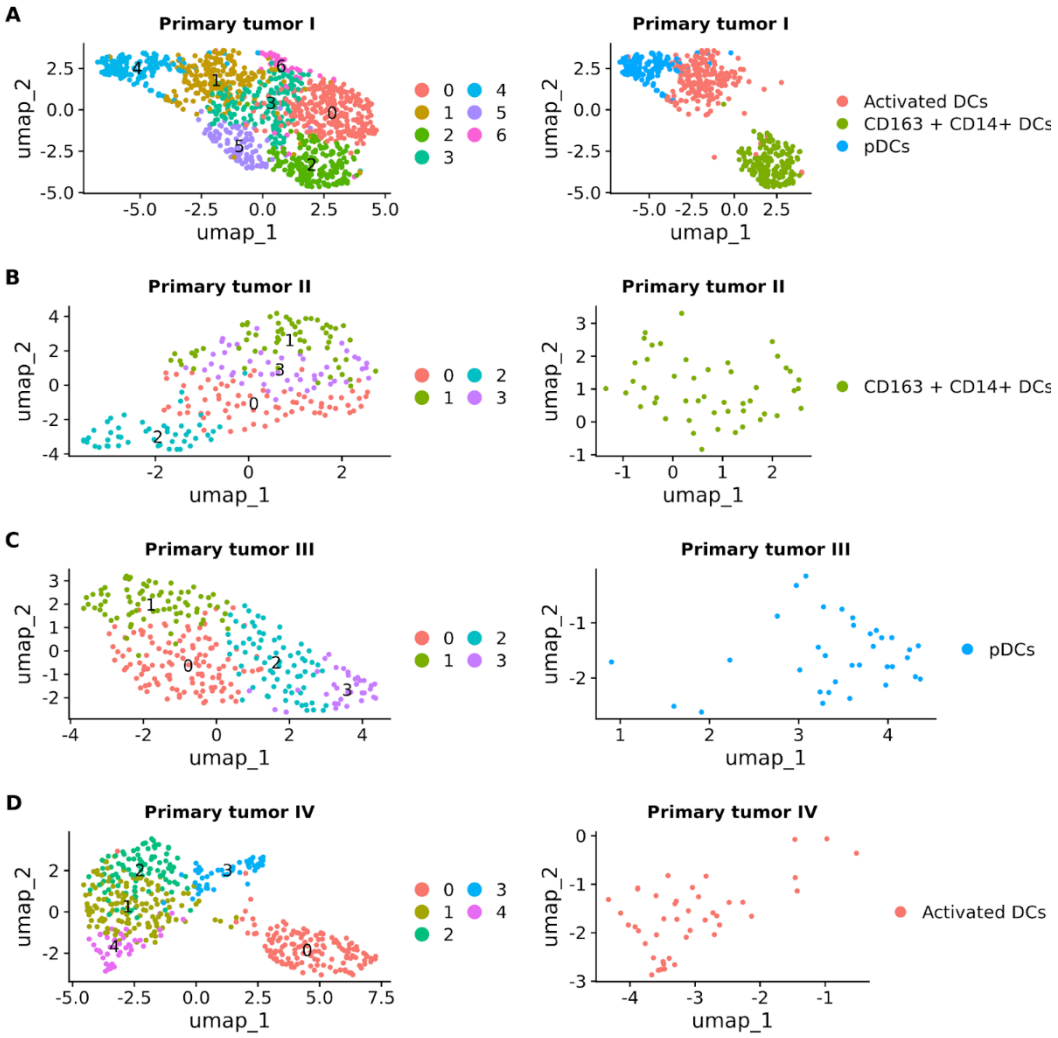

Figure 8. UMAP plots showing the subclusters of dendritic cells (DCs). **(A)** Primary tumor stage I. **(B)** Primary tumor stage II. **(C)** Primary tumor stage III. **(D)** Primary tumor stage IV. pDCs (plasmacytoid DCs).

2.7. Comprehensive Sub clustering of T and B Cells Reveals Stage-Specific Profiles

Afterward, we subclustered T cells obtained from both primary tumors and BM samples into distinct subsets: CD8+ T (naïve, cytotoxic, exhausted), naïve CD4+ T, T regulatory (Treg), T follicular helper, T helper 17, T helper 1, T helper 2, and gamma delta T.

In BM samples, a total of 13,818 T cells were further subclustered into 19 subclusters. Among these, subcluster 1 demonstrated a cytotoxic profile (*n*=2,670), subcluster 3 represented T helper 17 (*n*=1,257), subclusters 7 and 9 exhibited Treg characteristics (*n*=1,667), and subcluster 13 displayed features of exhaustion (*n*=315) **(Figure 9A)**. For primary tumor stage

I, 13,125 T cells underwent sub clustering, resulting in 13 distinct subclusters. Notably, subcluster 0 was identified as naïve CD4+ T (*n*=1,531), subclusters 2 and 7 as T helper 17 (*n*=2,500), and subclusters 3, 5, 8, and 9 as cytotoxic CD8+ T (*n*=3,893) **(Figure 9B)**. Moving to primary tumor stage II, 1,373 cells were clustered into 7 subclusters, with clusters 2, 4, and 5 classified as cytotoxic CD8+ T (*n*=567) **(Figure 9C)**. In primary tumor stage III, 4,865 T cells were subclustered into 10 subsets, where subclusters 0, 2, and 4 exhibited cytotoxic features (*n*=1,988), and subcluster 7 represented T helper 17 (*n*=396) **(Figure 9D)**. Lastly, for primary tumor stage IV, 2,696 cells were subclustered into 8 subclusters, with subclusters 0, 1, and 6 identified as CD8+ T cytotoxic (*n*=1,190) **(Figure 9E)**.

Shifting the focus to B cells, we reclassified them into various subsets, including GC B cells in the dark zone (DZ), GC B cells in the light zone (LZ), GrB-secreting cells, follicular B cells, mucosa-associated lymphoid tissue (MALT) B cells, and plasma cells. In BM samples, a total of 873 B cells were subclustered into 8 distinct subclusters. Notably, subcluster 0 was classified as follicular B cells (*n*=175), subcluster 1 as plasma cells (*n*=157), and subcluster 3 as MALT B cells **(Figure 10A)**. In primary tumor stage I, 324 B cells were subclustered into 7 subclusters. Specifically, cluster 2 represented GrB-secreting cells (*n*=52), subcluster 3 included GC B cells in the LZ (*n*=46), and subcluster 4 comprised MALT B cells (*n*=36) **(Figure 10B)**. Transitioning to primary tumor stage II, 133 B cells were subclustered into 5 subclusters, with subcluster 0 defined as GC B cells in the DZ (*n*=62) and subcluster 2 as MALT B cells (*n*=23) **(Figure 10C)**. In primary tumors stage III, 748 cells were subdivided into 6 subclusters, among which subclusters 0 and 1 were identified as plasma cells (*n*=464), and cluster 2 represented MALT B cells (*n*=137) **(Figure 10D)**. Finally, 39 B cells from primary tumor stage IV underwent sub clustering into 6 subclusters, with subclusters 2 and 4 classified as plasma cells (*n*=65) and B cells in the LZ (*n*=52), respectively **(Figure 10E).**

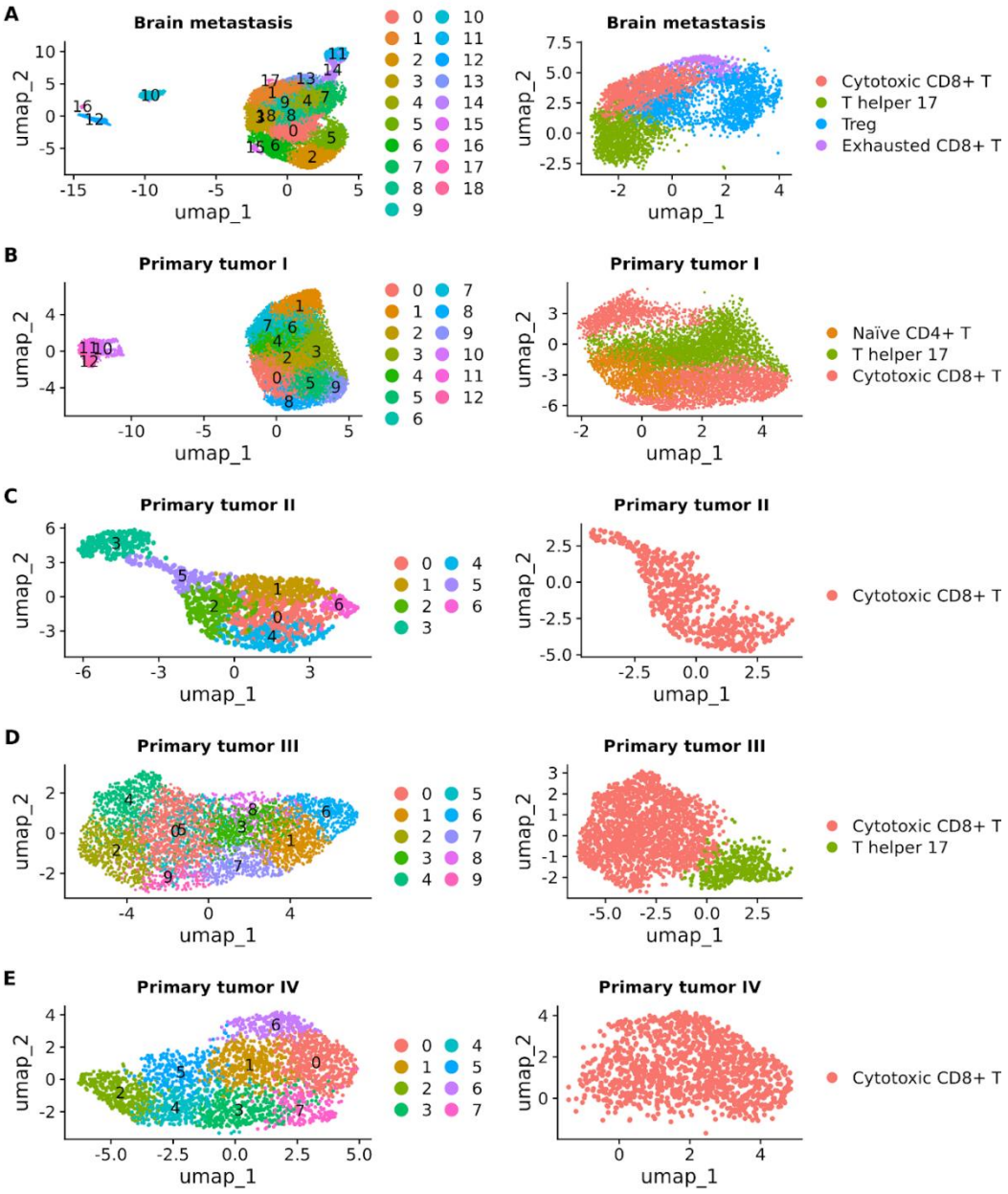

Figure 9. UMAP plots showing the subclusters of T cells. **(A)** Brain metastasis. **(B)** Primary tumor stage I. **(C)** Primary tumor stage II. **(D)** Primary tumor stage III. **(E)** Primary tumor stage IV. pDCs (plasmacytoid DCs).

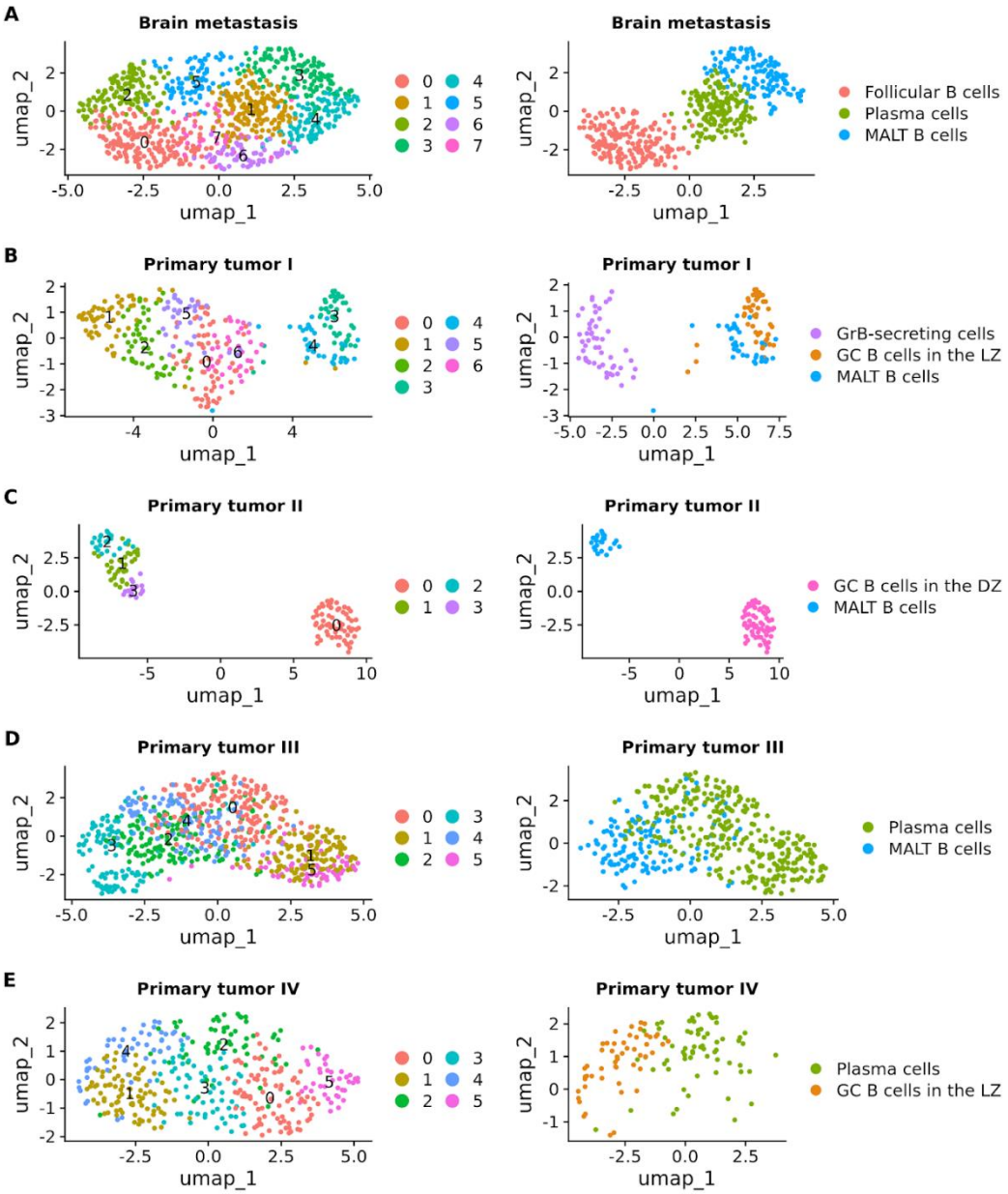

Figure 10. UMAP plots showing the subclusters of B cells. **(A)** Brain metastasis. **(B)** Primary tumor stage I. **(C)** Primary tumor stage II. **(D)** Primary tumor stage III. **(E)** Primary tumor stage IV. pDCs (plasmacytoid DCs).

3. Discussion

The TME is a complex ecosystem that surrounds the tumor, composed of a variety of elements including tumor cells, stromal cells, immune cells, extracellular matrix (ECM), blood vessels, chemokines and cytokines, and extracellular vesicles [21,22]. It plays a critical role in tumor development and progression, influencing various stages of tumorigenesis [23–25].

Recently, Hanahan revised the hallmarks of cancer and recognized the emerging participation of TME in cancer development [26]. It is well known that the TME is shaped by cancer cells to assist in developing cancer hallmarks, response to stress, stimulation, and treatment, and ultimately aiding in the survival and migration of tumor cells in an organism [27].

Because of their influence on tumor progression, the TME has received significant attention in the lung cancer literature in recent years, especially in the cancer therapy field [28,29]. Since the TME exerts a key influence on tumor cells and their behavior, therapeutic approaches for modulating the TME are promising [29]. Some of the strategies that have been explored include the inhibition of macrophage recruitment, reprogramming of tumor-associated macrophages (TAMs), depletion of TAMs, and engineering of TAMs [28,30,31]. Additionally, the effects of other treatment modalities, such as radiotherapy, chemotherapy, anti-EGFR treatment, or photodynamic therapy (PDT), combined with TAM-targeted therapy have also attracted attention [31]. Furthermore, targeting other components of the TME, such as tumor-infiltrating T cells [32], cancer-associated fibroblasts [33], and the ECM [34], has been investigated as a potential therapeutic approach.

While the TME and therapeutic strategies targeting it have been extensively researched in lung cancer [15,31,35], the focus has predominantly been on primary tumors, leaving a notable gap in studies related to BM. Additionally, many earlier clinical trials for immunotherapy in the context of metastatic disease excluded patients with brain lesions due to poor survival outcomes and concerns regarding the ability of drugs to cross the blood-brain barrier (BBB) [4]. These limitations underscore the urgent need for further investigations into the BM-TME.

The use of scRNA-seq technology has enabled the study of cellular and molecular heterogeneity of human tumors by distinguishing their different subpopulations [35]. This has proven crucial in understanding the mechanisms of tumor development and progression [36]. With the advancement of single-cell isolation techniques in the TME, high-quality scRNA-seq data, and new computational models for bioinformatics analyses, it has become possible to explore in more detail the complexity of the TME and to study intercellular communication and interactions between tumor cells and non-malignant cells [36,37].

Recent studies using scRNA-seq have explored the BM-TME leading to valuable insights into its diverse characteristics [17,38–40]. Nevertheless, these studies have been limited by small sample sizes, as BM samples are not always surgically removed. Samples of BM are rare, and obtaining samples from surgery is limited compared to other types of samples. This

rarity poses a challenge to studying BM. In this study, to address this challenge we integrated multiple datasets from scRNA-Seq analyses of BM-LUAD. To our knowledge, this is the first comprehensive study of its kind.

Our approach allowed us to obtain, after quality control, 79,729 cells from 23 samples of BM and 57,217 from 11 samples of primary tumors. These cells were cataloged into distinct cell types including immune and non-immune cells. The proportion of each cell type was quantified in both primary tumors and BM, revealing higher proportions of immune cells in both settings. Remarkably, our observations delineated distinct profiles of immunological infiltration into primary tumors and between BM and primary tumors. This aligns with existing literature indicating immunological differences between primary tumors and metastases in various cancer types, including breast cancer and melanoma [41,42]. For instance, a study found that immune cell infiltration of the primary tumor, not PD-L1 status, is associated with improved response to checkpoint inhibition in metastatic melanoma [43]. Furthermore, investigations have revealed that metastatic breast cancers are immunologically more inert than the corresponding primary tumors [41]. These dissimilarities in immune infiltration carry substantial implications for therapy responses and patient outcomes [43,44].

Notably, T cells were the dominant immune cell type in primary tumors, while microglia were the main cell type identified in BM. Similar to our results, in malignant gliomas, microglia are found as one of the main immune components of a tumor mass [45]. It is well known that microglia serve as the resident macrophages in the brain and are indispensable components of the brain microenvironment, participating in processes of innate immunity and maintaining central nervous system homeostasis [46,47]. In regular homeostatic conditions, microglia remain resting or quiescent, but they become activated in response to disease or injury, including tumor cell invasion [48]. These microglial cells release pro- and anti-inflammatory cytokines that aim to modulate the inflammatory scenario at the site of metastasis [49]. They are also involved in the formation of the pre-metastatic niche in the brain. Furthermore, microglia have been shown to interact with metastatic cancer and immune cells, and their functional plasticity can be modified by these interactions. Such interactions can impact the development of BM, including promoting tumor cell progression and influencing the metastatic colonization process [48,50–53].

According to our analysis using the ReactomeGSA R package analyse_sc_clusters function to quantify pathways in microglia [54] **(Supplementary Figure S1),** we found that

microglia have a marked upregulation of the COX reaction pathway, well-known for its association with regulating the inflammatory response [55]. Indeed, other studies have reported that, even in the initial stages, BMs are surrounded by a significant neuroinflammatory response mediated by activated astrocytes and microglia [4,48]. Inflammation is typically referred to as either acute or chronic [56]. Chronic inflammation contributing to cancer development via multiple mechanisms [56].

One potential mechanism is that chronic inflammation can generate an immunosuppressive microenvironment, allowing advantages for tumor formation and progression [56]. The immunosuppressive environment in certain chronic inflammatory diseases and solid cancers is characterized by infiltration of immune suppressor cells [56]. In a previous study [20], we demonstrated that BM is highly infiltrated by polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), which upregulate the IL-17 signaling pathway. In this previous study, supported by existing literature, we raised the hypothesis that PMN-MDSCs play an important role in creating an immunosuppressive microenvironment in BM.

It has been demonstrated that MDSCs are a targetable link between chronic inflammation and cancer [56], and contribute to cancer immune evasion by suppressing effector T cell activation, proliferation, trafficking, and viability; inhibiting NKs; and promoting activation and expansion of Treg cells [56–59]. Here, sub clustering of T cells **(Figure 9)** demonstrated that BM was the only group not primarily composed of T cell cytotoxicity, suggesting that MDSCs could be contributing to immune evasion in BM by suppressing effector T cell activation.

As mentioned before, in our previous study we found PMN-MDSCs enriched in interleukin-17 (IL-17) [20]. IL-17 is a pro-inflammatory cytokine that has been implicated in the recruitment of MDSCs contributing to immune suppression and tumor progression [60]. The interaction between IL-17 and MDSCs is influenced by the local and systemic levels of interleukin-1 (IL-1), which can promote the accumulation of MDSCs in the tumor microenvironment [61]. IL-17 is produced by T helper 17 (Th17) cells [62]. Th17 are effector cells that promote neuroinflammation [62]. We have previously shown that BM is enriched in the CD163 + CD14+ DC subset, which possesses a strong Th17 polarizing capacity, as evidenced by the pro-Th17 gene signature [20]. It was demonstrated that the generation of Th17 cells requires certain pro-inflammatory cytokines such as interleukin-23 (IL-23), which

is primarily secreted by antigen-presenting cells (APCs) like DCs, macrophages, and B cells [63]. However, in the context of neuroinflammation and neurodegenerative diseases, microglia have also been shown to produce IL-23 [64–67].

Our data showed that both microglia and DCs upregulated JAK-STAT signaling after interleukin-12 stimulation **(Supplementary Figure S2)**. IL-23 utilizes orthologs of gp130 as part of their receptor complex to signal through the JAK/STAT pathway [68,69]. A study using cell lines with brain-metastatic tropism showed that the polarized phenotype of microglia via JAK2/STAT3 signaling has been implicated in promoting BM of NSCLC, by enhancing colonization [52].

Collectively, these results suggest that activated microglia, CD14+ DC, Th17, and PMN-MDSCs are closely interconnected in the context of BM, encompassing chronic inflammation and an immunosuppressive environment. However, there is still a need for further investigation to gain a deeper understanding of the specific roles played by microglia, T cells, and MDSCs in shaping the immunosuppressive BM-TME as well as their exact contributions to the progression of BM.

By using CellphoneDB to explore intercellular communications through receptor-ligand relationship pairs, we discovered that microglia primarily interact with oligodendrocytes and endothelial cells. One significant interaction was identified between *DLL4* and *NOTCH4*, which demonstrated a relevant association from endothelial cells to microglia, and from microglia to oligodendrocytes. The Notch signaling pathway, including DLL4-Notch4 interactions, has been associated with vasculogenic mimicry, tumor recurrence, and prognosis in NSCLC and other malignancies [70,71]. *DLL4* and *NOTCH4* have been investigated for their roles in promoting metastasis and cancer stem cell activities, and their downregulation has been linked to reduced metastatic burden and inhibition of cancer stem cells [72]. Therefore, considering both the existing literature and our findings, the *NOTCH4* and *DLL4* genes emerge as potential therapeutic targets for the treatment of BM, and further exploration of their functions in this context is warranted.

Additionally, we found another notable interaction between *VEGFC* and *KDR*, revealing a significant association between microglia to oligodendrocytes ($p = 0.029$). The expression of VEGF and its receptor, KDR, has been correlated with vascularity, metastasis, and proliferation of human colon cancer [73]. Targeting the VEGF pathway, including *VEGF* and *KDR*, has been explored as a potential therapeutic strategy for brain tumors [73].

T cells were found to dominate the immune landscape in primary tumors **(Figure 5A)**. The observed high percentage of T cells among immune-infiltrating cells in NSCLC is in accordance with previous reports [14,20]. We further subclustered these T cells into subtypes, including cytotoxic CD8+ T cells, T helper 17, and naïve CD4+ T cells in primary tumors, and cytotoxic CD8+ T cells, T helper 17, naïve CD4+ T cells, and exhausted CD8+ T cells in BM samples. Cytotoxic CD8+ T cells were the only subtype identified in primary tumors stages II and IV, and the most abundant subtype in stage III.

Tumor-infiltrating T cells, particularly T cytotoxic cells, play an indispensable role in the immune response against tumor cells [74,75]. DCs capture tumor-associated antigens (TAAs) and migrate to lymph nodes, where they present TAAs on major histocompatibility complex (MHC) molecules to naïve T cells, triggering the activation of TAA-specific CD4+ helper T cells or CD8+ cytotoxic T cells, via MHC class II or MHC class I, respectively [76–78]. The activation of T cells is highly regulated, requiring recognition of antigens in the context of appropriate MHC molecules [79]. Several studies have shown that the diversity of the HLA repertoire can directly affect the strength of the anti-tumor immune response [80]. In various types of cancer, including lung cancer, the MHC-I genotype and tumor mutational burden have been found to be predictors of immunotherapy response [81]. Additionally, MHC-II signature has been found to be associated with anti-tumor immunity and can predict anti-PD-L1 therapeutic response of patients with bladder cancer [82].

In this study, we have observed that several genes within the HLA complex are downregulated in BM tissue in comparison to primary tumor stage IV **(Figure 7)**. It is common for cancers to experience HLA downregulation [83]. Tumor cells may escape T cell attack through HLA downregulation, limiting HLA-dependent immunotherapy to some extent [84,85]. Yang et al. conducted a study that showed how HLA-I downregulation in glioma stem cells was linked with abnormal Wnt/β-catenin activity [86]. In progressing metastases of melanoma patients treated with ipilimumab, HLA class I downregulation was most pronounced in progressing metastases from non-responding patients [87]

Collectively, these results suggest that the downregulation of HLA genes in BM, particularly in the context of lung adenocarcinoma, may serve as an immune evasion mechanism. Our findings illuminate the complexity of molecular and cellular dynamics of BM-LUAD. Recognizing and understanding such mechanisms is essential for addressing the challenges associated with T-cell-based immunotherapy in the treatment of BM-LUAD.

4. Materials and Methods

4.1. Data collection

In this study, we conducted comprehensive analyses by utilizing multiple datasets sourced from the Gene Expression Omnibus (GEO) (available online at: https://www.ncbi.nlm.nih.gov/gds/, accessed on 30 November 2023). The specific datasets used are detailed in **Table 1**.

4.2. Pre-processing and quality control of scRNA-seq data

All analyses were carried out in R 4.2.1. The pre-processing and quality control were conducted as previously described [20]. Briefly, Seurat objects were created from individual expression matrices using the "Seurat" R package (version 4.0.2) [88,89]. Cells expressing fewer than 200 or more than 9000 genes, along with those exhibiting a mitochondrial gene percentage exceeding 20%, were excluded. Furthermore, genes expressed in fewer than 3 cells were also excluded. The remaining cells underwent the normalization of gene expression matrices using the NormalizeData function in the Seurat package. To identify the genes that exhibit the highest cell-to-cell variation, Seurat FindVariableFeatures was used to select the top 2000 genes.

4.3. Dimensionality reduction, clustering, and cell type annotations

The datasets were integrated and the batch effects were removed by canonical correlation analysis and mutual nearest neighbors-anchors using the functions SelectIntegrationFeatures, FindIntegrationAnchors, and IntegrateData from the Seurat package. Following that, we applied data scaling with the Seurat ScaleData function and linear dimensional reduction through Principal Component Analysis (PCA) using the RunPCA function. To visualize both cells and features defining the PCA functions such as DimPlot, VizDimLoadings, and ElbowPlot were employed. The optimal dimensionality of the dataset was determined using the ElbowPlot function. Cell visualization was performed using uniform manifold approximation and projection (UMAP) through the RunUMAP function. We utilized the FindAllMarkers function to identify the differentially expressed genes (DEGs) in each subset

or subcluster. The criteria for DEGs were *p*. adj < 0.05 (Wilcoxon rank-sum test) and |log2 FC| > 1. Then, we annotated clusters and subclusters using the CellMarker2.0 database [90] and after that, we validated the annotation manually using specific cell surface markers previously described in the literature (**Supplementary Table S16)**. Only genes expressed in over 25% of cells with at least a 0.25-fold difference were considered for each cluster and subcluster.

4.4. scRNA-Seq Pathway Analysis

To identify the pathways enriched by cell types we used the analyze_sc_clusters function and extracted the results through the pathways function from the "ReactomeGSA" package (version 1.12.0) [54].

4.5. Differential expression analysis of immune cells and enrichment analysis

To identify immune cells' DEGs between BM and primary tumors the FindMarkers function from the Seurat package. The significance of the difference was determined by using the Wilcoxon rank-sum test with the Bonferroni correction. Genes with |log2 FC| > 1, and an adjusted *p*-value < 0.05 were considered DEGs. The enrichment analysis was conducted as previously described [20]. Briefly, we utilized the enrichGO() function from the R package clusterProfiler (version 4.0.5) [91] to investigate Gene Ontology (GO). To perform the GO enrichment analysis, we obtained the GO annotation file from Gene Ontology (available online at: http://geneontology.org/, accessed on 1 October 2021). For the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, we used the enrichKEGG() function from clusterProfiler. In all analyses, we controlled the false discovery rate (FDR) by adjusting the *p*-value using the Benjamini–Hochberg method. We considered categories with a cutoff of *p*. adj < 0.05 as significant. To visualize the results, we used the Ggplot2 and GOplot packages [92,93].

4.6. Cell communication analysis

The analysis of cell communication was conducted using CellPhoneDB (version 2.1.7) [94], a publicly available database of receptor-ligand interactions. The cell matrix was normalized using Seurat Normalization. The significance of cell communication ($p < 0.05$) and

the significant mean were calculated based on the interaction. The results were visualized using the CCPlotR package (version 0.99.3) [95].

5. Conclusions

We have presented a detailed overview of the TME of BM-LUAD and primary tumor. By integrating multiple scRNA-seq datasets, we have identified unique immunological infiltration profiles in primary tumors and BM. Our analysis shows that T cells dominate in primary tumors, while microglia are the primary immune cells in BM, emphasizing the importance of the brain microenvironment in shaping the BM-TME.

Our study also highlights the role of chronic inflammation in BM and focuses on PMN-MDSCs and their association with IL-17 signaling. We have discussed the interconnected roles of microglia, T cells, and MDSCs in chronic inflammation within the BM-TME.

We have used CellphoneDB to analyze intercellular communication and have identified significant interactions between microglia, endothelial cells, and oligodendrocytes. This suggests potential therapeutic targets like Notch4 and DLL4. Additionally, the VEGF pathway involving *VEGFC* and *KDR* has shown associations with microglia and oligodendrocytes, providing insights into vascularization and proliferation in BM.

Finally, we identified a downregulation of HLA genes in BM, indicating a potential immune evasion mechanism. Understanding these mechanisms is crucial for addressing challenges associated with T-cell-based immunotherapy in BM-LUAD.

This study provides a crucial basis for researching the molecular mechanisms and targeted therapy of LUAD. The findings emphasize the necessity for continued research to understand the complexities of the BM-TME, which can help pave the way for targeted therapeutic interventions and improved patient outcomes.

Supplementary Materials: Figure S1: Functional enrichment analysis for the identified cell types in brain metastasis using the "ReactomeGSA" package; Figure S2: Functional enrichment analysis showing only JAK-STAT signaling after Interleukin-12 stimulation pathway for the identified cell types in brain metastasis; Figure S3: Functional enrichment

analysis for the identified cell types in primary tumor I using the “ReactomeGSA” package; Figure S4: Functional enrichment analysis for the identified cell types in primary tumor II using the “ReactomeGSA” package; Figure S5: Functional enrichment analysis for the identified cell types in primary tumor III using the “ReactomeGSA” package; Figure S6: Functional enrichment analysis for the identified cell types in primary tumor IV using the “ReactomeGSA” package; Table S1: Proportion of each cell type in both primary tumors and brain metastasis; Table S2: Ligand-receptor pairs and molecular interactions among the major cell types for brain metastasis samples; Table S3: Ligand-receptor pairs and molecular interactions among the major cell types for primary tumors stage TI; Table S4: Ligand-receptor pairs and molecular interactions among the major cell types for primary tumors stage TII; Table S5: Ligand-receptor pairs and molecular interactions among the major cell types for primary tumors stage TIII; Table S6: Ligand-receptor pairs and molecular interactions among the major cell types for primary tumors stage TIV; Table S7: Differentially expressed genes in T cells; Table S8: Differentially expressed genes in Macrophages; Table S9: Differentially expressed genes in NK cells; Table S10: Differentially expressed genes in neutrophils; Table S11: Differentially expressed genes in Dendritic cells (DCs); Table S12: Differentially expressed genes in B cells; Tabel S13: Differentially expressed genes in Mast cells; Table S14: Enrichment analysis interaction network from the Kyoto Encyclopedia of Genes and Genomes (KEGG); Table S15: Gene ontology (GO) enrichment analysis; Table S16: List of the markers to assign the cell types and subtypes.

Author Contributions

V.G.P.S., and P.P.R. initiated the project. V.G.P.S. designed, researched, analyzed, and wrote the topics covered in the article. V.G.P.S. and N.T. designed the figures. N. T., P.P.R., and W.L.L. reviewed and edited the manuscript. All authors contributed to manuscript preparation and approved the final version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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Conflicts of Interest

The authors declare no conflict of interest.

5. Considerações finais

O câncer de pulmão continua a ser a principal causa de mortalidade por câncer em todo o mundo. O desafio crucial enfrentado na abordagem deste câncer é a detecção tardia, uma vez que os pacientes geralmente permanecem assintomáticos nas fases iniciais do desenvolvimento da doença. Esta realidade resulta em diagnósticos frequentes em estágios avançados, associados ao insucesso terapêutico e à baixa sobrevida dos pacientes. Apesar dos avanços recentes nas terapias multimodais e direcionadas, os tratamentos disponíveis para MC em pacientes com câncer de pulmão são limitados, beneficiando apenas alguns e apresentando custos elevados.

Neste contexto, a urgência de estudos moleculares em larga escala é evidente, visando a identificação de biomarcadores que possam direcionar o desenvolvimento de terapias com alvos moleculares. Propomos, assim, a investigação do transcriptoma das MC em pacientes diagnosticados com adenocarcinoma de pulmão, utilizando abordagens computacionais integradas para identificar biomarcadores com potencial aplicação clínica.

O primeiro capítulo oferece uma revisão abrangente do estado da arte no estudo das MC de adenocarcinoma de pulmão, abordando eventos-chave na formação da MC, influência do microambiente tumoral e determinantes moleculares da progressão. Destacamos os avanços no diagnóstico molecular da MC, incluindo biópsias líquidas, e discutimos estratégias de tratamento inovadoras.

No segundo capítulo, integramos RNA-Seq total, microarray e scRNA-Seq para identificar redes regulatórias e vias moleculares. Descobrimos 102 genes desregulados, indicando a importância do sistema imunológico no desenvolvimento da MC. Nosso estudo revelou um padrão de expressão gênica específico nas MC e a presença de células dendríticas do tipo CD163 + CD14+ que apresentaram expressão aumentada de genes HLA e enriqueceram vias de processamento e apresentação de antígenos. Além disso, essas células apresentaram forte capacidade de polarização Th17, como evidenciado pela assinatura do gene pró-Th17. Identificamos também a infiltração de células supressoras polimorfonucleares derivadas de mieloides (PMN-MDSCs).

No terceiro capítulo, exploramos o microambiente tumoral das MC, destacando a predominância de células da microglia. A microglia é identificada como um componente essencial do microambiente cerebral, desempenhando papel na modulação do cenário

inflamatório e na formação do nicho pré-metastático no cérebro. Mostramos *(in silico)* que a microglia regula positivamente a via de reação COX, associada à resposta inflamatória. A interação entre IL-17 e PMN-MDSCs é discutida, sugerindo um ambiente imunossupressor na MC. Os resultados indicam que microglia ativada, células dendríticas CD163+ CD14+, Th17 e PMN-MDSCs estão interligados na formação da MC, com implicações na inflamação crônica e ambiente imunossupressor.

Na figura abaixo resumimos os principais achados deste trabalho e ilustramos a intricada rede de componentes imunológicos dentro do microambiente tumoral da MC de adenocarcinoma de pulmão.

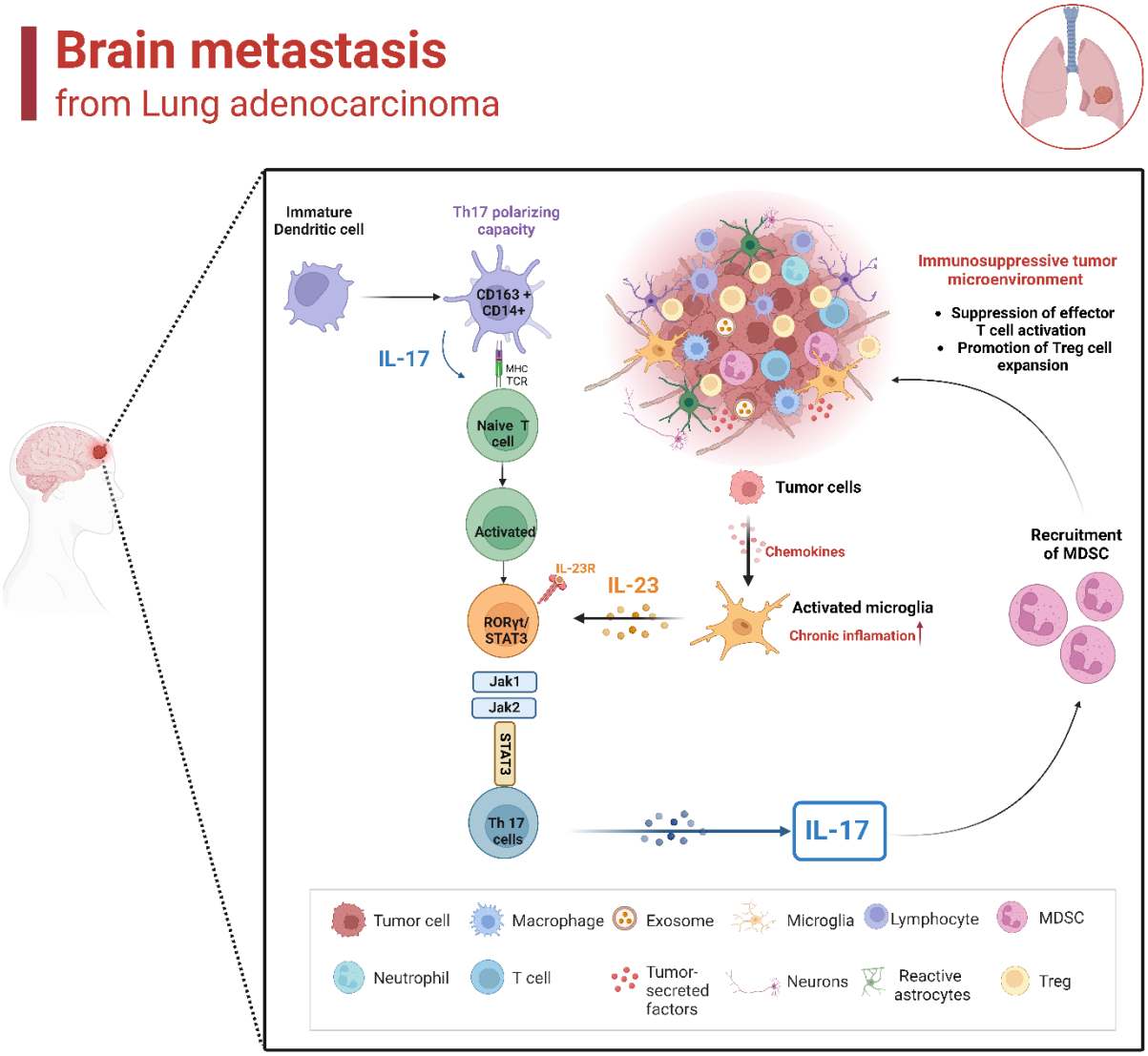

Figura 1. Esta figura ilustra a intricada rede de componentes imunológicos dentro do microambiente de metástase cerebral (MC). As microglias, identificadas como elementos imunológicos mais abundantes na MC, são ativadas em resposta a uma série de citocinas e quimiocinas liberadas pelas

células tumorais. As células microglias ativadas, por sua vez, regulam positivamente uma série de vias associadas à inflamação (por exemplo, a via COX) e liberam citocinas como a interleucina-23 (IL-23), uma proteína sinalizadora do sistema imunológico classificada como citocina pró-inflamatória. A IL-23 desempenha um papel fundamental no crescimento tardio e na maturação das células Th17, ativando seu receptor IL-23R. As células Th17 diferenciadas primárias (ainda não maduras), expressam fatores de transcrição específicos, como RORγt e STAT3. RORγt é o principal regulador da diferenciação das células Th17. A IL-23, secretada pela microglia ativada, liga-se aos receptores IL-23 na superfície celular das células Th17 diferenciadas primárias, ativando os JAKs associados, principalmente JAK2. Os JAKs ativados fosforilam o receptor e, em seguida, o STAT3 se liga ao receptor fosforilado. Os JAKs também fosforilam o STAT3, ativando-o. O STAT3 fosforilado move-se para o núcleo da célula, onde influencia a expressão de genes específicos relacionados à maturação de Th17. Essa cascata de sinalização é fundamental para as respostas imunológicas mediadas pela IL-23, incluindo a ativação das células Th17 e a produção de citocinas pró-inflamatórias, como a interleucina-17 (IL-17). A IL-17 é uma citocina pró-inflamatória implicada no recrutamento de células supressoras polimorfonucleares derivadas de mieloides (PMN-MDSCs) para o microambiente tumoral. As MDSCs, conhecidas como uma ligação entre a inflamação crônica e o câncer, contribuem para a evasão imunológica, suprimindo a ativação de células T efetoras e promovendo a expansão de células Treg. Além disso, as células dendríticas do tipo CD163 + CD14+, com capacidade de polarização Th17, secretam IL-17, induzindo a diferenciação de células T em Th17. Nossas descobertas iluminam a complexidade das interações que abrangem o transcriptoma codificador com a modulação do sistema imunológico na metástase cerebral de adenocarcinoma de pulmão.

6. Conclusões

Este trabalho fornece análises detalhadas do microambiente tumoral (TME) das MC de adenocarcinoma de pulmão e revela insights importantes sobre os mecanismos subjacentes à formação das MC e estratégias terapêuticas potenciais. No primeiro estudo, identificamos 102 genes com expressão alterada no ambiente cerebral, associados a moléculas de adesão celular, sinalização de quimiocinas e vias de diferenciação celular. Destacaram-se como genes-chave *CD69* e *GZMA*, relacionados à regulação imunológica. O segundo estudo analisou o TME tanto no tumor cerebral metastático quanto no tumor primário, destacando perfis únicos de infiltração imunológica. Células T predominaram nos tumores primários, enquanto as microglia foram proeminentes no ambiente cerebral metastático. A inflamação crônica foi associada às células dendríticas CD163 + CD14+, enfatizando os papéis interconectados da microglia, células T e MDSCs na inflamação. A análise de comunicação intercelular identificou interações importantes entre microglia, células endoteliais e oligodendrócitos, sugerindo potenciais alvos terapêuticos. A regulação negativa de genes HLA na MC indica um mecanismo potencial de evasão imunológica. No geral, essas descobertas ressaltam a importância do sistema imunológico na MC de adenocarcinoma de pulmão, e abordar o sistema imunológico surge como uma estratégia promissora para o tratamento da MC. Além disso, os resultados deste trabalho fornecem informações valiosas sobre os mecanismos moleculares envolvidos na formação da MC em adenocarcinoma de pulmão. Futuramente, esperamos que nossos dados contribuam com o desenvolvimento de novas estratégias de tratamento e detecção precoce da MC.

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8. Materiais suplementares

8.1 Figuras suplementares

Capítulo 2.

Acesso: https://www.mdpi.com/article/10.3390/cancers15184526/s1

8.2 Tabelas suplementares

Capítulo 2.

Acesso: https://www.mdpi.com/article/10.3390/cancers15184526/s1

Capítulo 3.

Acesso:
https://drive.google.com/drive/u/0/folders/1IRfN6WMTfEs3OPTszeZ_89v1jrJA1oBB

Anexos

Anexo 1.

Publicações como primeira autora durante o doutoramento.

1. **Souza VGP***, Forder A, Pewarchuk M, Telkar N, Araujo R, Stewart G, Vieira J, Reis PP, Lam WL (2023). The Complex Role of the Microbiome in Non-Small Cell Lung Cancer Development and Progression. Cells; 12(24), 2801.

2. **Souza VGP***, Forder A, Telkar N, Stewart G, Carvalho RF, Mur LAJ, Lam WL, Reis PP (2023). Identifying New Contributors to Brain Metastasis in Lung Adenocarcinoma: A Transcriptomic Meta-Analysis. Cancers; 15(18), 4526.

3. **Souza VGP***, Forder A, Brockley LJ*, Pewarchuk ME, Telkar N, de Araújo RP, Trejo J, Benard KH, Seneda AL, Minutentag IW, Erkan M, Stewart GL, Hasimoto EN, Garnis C, Lam WL, Martinez VD, Reis PP (2023). Liquid Biopsy in Lung Cancer: Biomarkers for the Management of Recurrence and Metastasis. International Journal of Molecular Sciences; 24(10):8894.

4. **Souza VGP***, Araújo RP, Santesso MR, Seneda AL, Minutentag IW, Felix TF, Filho PTH, Pewarchuk ME, Brockley LJ, Marchi FA, Lam WL, Drigo SA, Reis PP (2023). Advances in the Molecular Landscape of Lung Cancer Brain Metastasis. Cancers; 24;15(3):722.

5. Brockley LJ*, **Souza VGP***, Forder A, Pewarchuk ME, Erkan M, Telkar N, Benard KH, Trejo J, Stewart MD, Stewart GL, Reis PP, Lam WL, Martinez VD (2023). Sequence-Based Platforms for Discovering Biomarkers in Liquid Biopsy of Non-Small-Cell Lung Cancer. Cancers; 15(8), 2275.

Publicações como coautora durante o doutoramento.

1. Minutentag IW*, Seneda Al, Barros-Filhos MC, Carvalho M, **Souza VGP**, Hasimoto CN, Moraes MPT, Marchi FA, Lam WL, Reis PP, Drigo SA (2023). Discovery of novel miRNAs in colorectal cancer: potential biological roles and clinical utility. Non-Coding RNA; 9(6), 65.

2. Botta FP*, Rocha LA, **Souza VGP**, Reis PP, Lima EO, Ferrasi AC, Fernandes AY, Zanini MA, Hamamoto-Filho PT (2023). Survival in patients undergoing surgical resection for brain metastasis from lung cancer and utility of different prognostic scales. Neurosurgical Review; 46(1):184.

3. Camargo BC*, **Souza VGP**, Lapa RML, Reis PP, Oliveira RA (2023). A decision tree-based classifier compares three data analysis methods for the identification of miRNAs associated with early-stage lung cancer. Revista Foco (Interdisciplinary Studies), 16(5), e2031.

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