# Targeting the stressed tumor niche of glioblastoma: Tumor microenvironment and surface antigens

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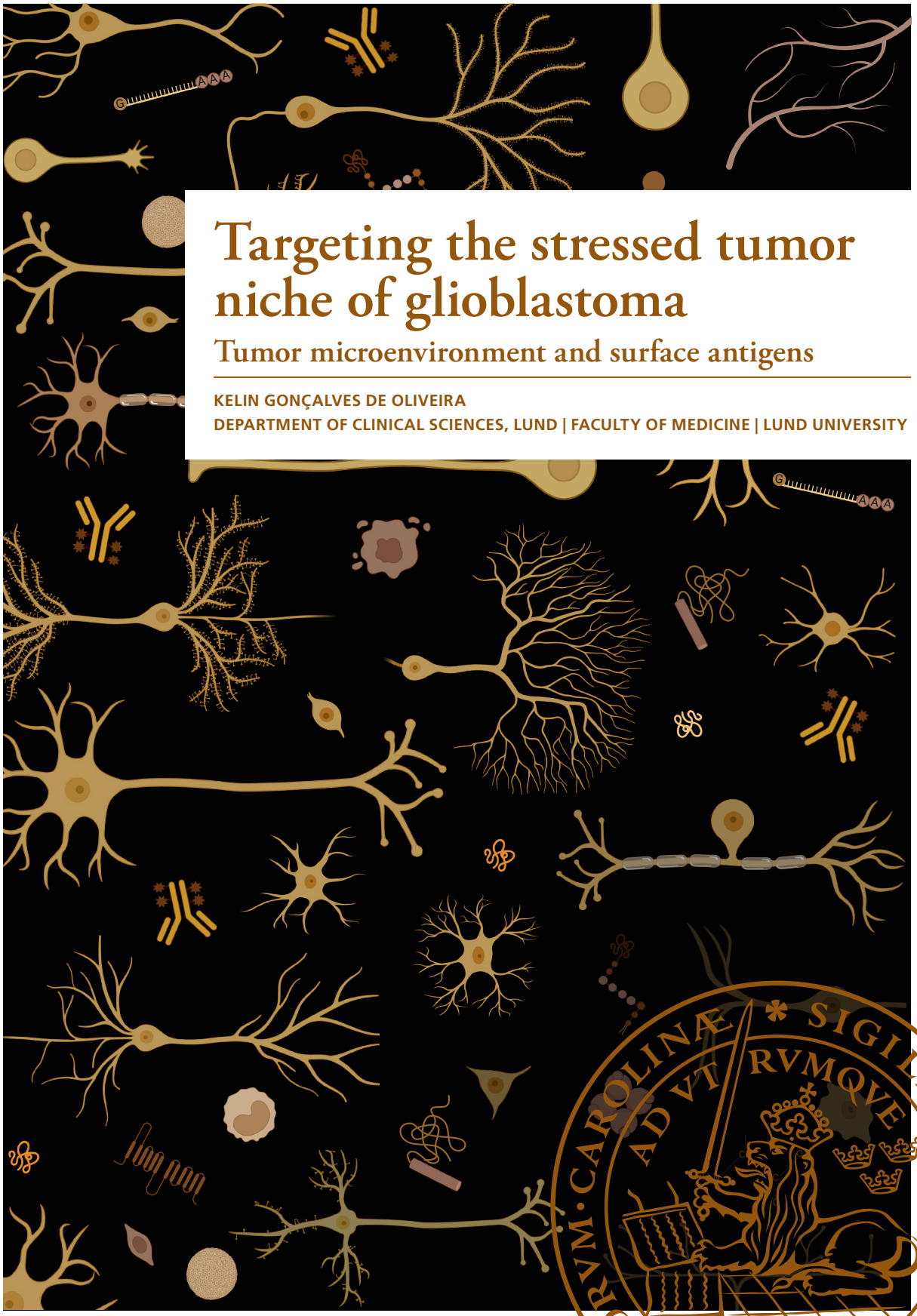

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## Tumor microenvironment and surface antigens

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**Abstract**

Cancer is a complex and devastating disease that affects millions of people worldwide. This malignancy is defined by the uncontrolled growth and division of abnormal cells in the body, which can invade and destroy healthy tissues. One important aspect of cancer biology is the tumor microenvironment (TME), a dynamic network of cancerous, stromal, and immune cells interacting with blood vessels, extracellular matrix, and soluble molecules within the tumor mass. The TME is known to play a crucial role in cancer progression and response to therapy.

Glioblastoma (GBM) is an aggressive type of brain cancer that arises from glial cells, *i.e.* non-neuronal support cells responsible for maintaining homeostasis. Among other brain malignancies, GBM is the most common type of primary brain tumor diagnosed in adults and, despite efforts, patients usually face poor prognosis and limited treatment options. One of the features of the GBM TME known to contribute to poor patient outcomes is hypoxia, which arises when the tumoral mass outgrows its own blood supply. Hypoxia triggers the selection of aggressive cancer cell populations that respond differently to available treatments, ultimately resulting in tumor progression, invasion, and angiogenesis.

Understanding the interplay between cancer cells and the TME is crucial for developing effective therapeutic strategies for GBM. Recently, the targeting of TME components has emerged as a promising approach for improving the treatment outcomes for patients. Therefore, this thesis dives into the TME of GBMs, with a particular focus on the role of hypoxia in promoting tumor aggressiveness. In **Paper I**, we investigated the role of lipids in the hypoxic response of GBM cells and found that lipid-loaded areas in patient-derived GBM tissues presented with increased hypoxic signaling through *e.g.* VEGF and HGF. This was associated with increased macrophage infiltration and angiogenesis. In **Paper II**, we presented the Tumor Surfaceome Mapping (TS-MAP) platform, a new strategy for the efficient profiling of the tumor surfaceome and endocytome. The TS-MAP platform is a valuable tool for the discovery of targetable proteins from intact freshly resected tumors or cultured cells. In a follow-up study, presented in **Paper III**, the TS-MAP protocol was applied to transcriptionally divergent primary GBM cell models submitted to normoxia or hypoxia, for the identification of hypoxia-induced endocytosed proteins. We confronted identified proteins with antibody-drug-conjugates, as a potential strategy to target the hypoxic, treatment-resistant tumor niche. Lastly, reconnecting with the findings of the first paper, **Paper IV** (unpublished) characterizes lipid-loaded macrophages, defined as Tumor Associated Foam Cells (TAFs), within the hypoxic area of GBMs. We propose that the uptake of lipid particles present in the TME induces the emergence of TAFs, distinguished by low phagocytic capacity, as well as pro-tumorigenic and pro-angiogenic phenotypes.

By unraveling the aforementioned complexities, this thesis contributes to an increased academic understanding of the TME in GBM and provides tools for the identification of potential therapeutic targets.

**Key words:** Glioblastoma, Tumor Microenvironment, Hypoxia, Surface Proteins, Lipids.

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**Cover illustration by Kelin Gonçalves de Oliveira: Academic Tapestry**.
Digital composite. Visual exploration showcasing a few "characters" from Papers I-IV. Can you name them all? (Made with BioRender).



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*To my family.*
*Without whom I wouldn't have learnt how to dare.*
*Amo vocês.*

*"Understand it well as I may, my comprehension can only be an infinitesimal fraction of all I want to understand."*
*- Ada Lovelace*

# Table of Contents

# Abstract

Cancer is a complex and devastating disease that affects millions of people worldwide. This malignancy is defined by the uncontrolled growth and division of abnormal cells in the body, which can invade and destroy healthy tissues. One important aspect of cancer biology is the tumor microenvironment (TME), a dynamic network of cancerous, stromal, and immune cells interacting with blood vessels, extracellular matrix, and soluble molecules within the tumor mass. The TME is known to play a crucial role in cancer progression and response to therapy.

Glioblastoma (GBM) is an aggressive type of brain cancer that arises from glial cells, *i.e.* non-neuronal support cells responsible for maintaining homeostasis. Among other brain malignancies, GBM is the most common type of primary brain tumor diagnosed in adults and, despite efforts, patients usually face poor prognosis and limited treatment options. One of the features of the GBM TME known to contribute to poor patient outcomes is hypoxia, which arises when the tumoral mass outgrows its own blood supply. Hypoxia triggers the selection of aggressive cancer cell populations that respond differently to available treatments, ultimately resulting in tumor progression, invasion, and angiogenesis.

Understanding the interplay between cancer cells and the TME is crucial for developing effective therapeutic strategies for GBM. Recently, the targeting of TME components has emerged as a promising approach for improving the treatment outcomes for patients. Therefore, this thesis dives into the TME of GBMs, with a particular focus on the role of hypoxia in promoting tumor aggressiveness. In **Paper I**, we investigated the role of lipids in the hypoxic response of GBM cells and found that lipid-loaded areas in patient-derived GBM tissues presented with increased hypoxic signaling through *e.g.* VEGF and HGF. This was associated with increased macrophage infiltration and angiogenesis. In **Paper II**, we presented the Tumor Surfaceome Mapping (TS-MAP) platform, a new strategy for the efficient profiling of the tumor surfaceome and endocytome. The TS-MAP platform is a valuable tool for the discovery of targetable proteins from intact freshly resected tumors or cultured cells. In a follow-up study, presented in **Paper III**, the TS-MAP protocol was applied to transcriptionally divergent primary GBM cell models submitted to normoxia or hypoxia, for the identification of hypoxia-induced endocytosed proteins. We confronted identified proteins with antibody-drug-conjugates, as a potential strategy to target the hypoxic, treatment-resistant tumor niche. Lastly, reconnecting with the findings of the first paper, **Paper IV** (unpublished) characterizes lipid-loaded macrophages, defined as Tumor Associated Foam Cells (TAFs), within the hypoxic area of GBMs. We propose that the uptake of lipid particles present in the TME induces the emergence of TAFs, distinguished by low phagocytic capacity, as well as pro-tumorigenic and pro-angiogenic phenotypes.

By unraveling the aforementioned complexities, this thesis contributes to an increased academic understanding of the TME in GBM and provides tools for the identification of potential therapeutic targets.

# Popular summary in English

Cancer is a disease that affects millions of people all over the world. It arises when abnormal cells in our body start growing and spreading uncontrollably, attacking healthy tissues along the way. In this thesis, the focus is given to the investigation of **glioblastoma (GBM)**, an aggressive and yet incurable type of brain cancer diagnosed in adults. The studies here presented were developed to help improve the current knowledge about this tumor type and to present potential tools to aid the fight against this malignancy.

In a neighborhood metaphor, we can regard the healthy brain cells as the native residents of a busy community. Comparatively, the emergence of a GBM tumor would represent the arrival of aggressive burglars that rapidly expand and skillfully avoid surveillance. As expected, their presence disrupts the balance, rendering the community less functional over time. The cancer cells establish chaotic interactions amongst themselves and other tumor-surrounding structures (*e.g.* blood vessels, immune cells, and molecules), forming a disruptive ecosystem known as the **tumor microenvironment (TME)**. In our analogy, different components of the TME can be compared to essential things surrounding us, and they contribute directly to cancer maintenance and growth. For example, the **extracellular matrix (ECM)**, *i.e.* a network of proteins and carbohydrates, provides the structural support the tumor requires, much like the steel found within constructions. Such structure is maintained and modified by engineer cells known as **stromal cells**, which lay connective tissue to shape the TME according to the tumor's demand. The role of roads is represented by **blood vessels** since these allow cells, metabolites, and information to transit within the tumoral and healthy compartments. Moreover, symbolizing the local authorities, **immune cells** play a vital role in surveillance and defense. Within the TME, these authorities are seen either fighting cancer cells or being blinded by their tricks. Lastly, as the tumor bulk grows larger, the formation of new blood vessels (roads) is not able to keep up with the ever-increasing demand for nutrient delivery. This culminates in the emergence of poorly perfused zones, characterized by the deprivation of essential nutrients and extracellular acidification (*i.e.* low pH) due to the accumulation of waste products. Consequently, a novel feature of the TME appears: the **Necrotic** region, defined by clusters of dead cells that succumbed to the dangerous milieu.

By visualizing the TME as a hypothetical neighborhood, we can begin to understand the complex dynamics between the different components of the tumor. This knowledge is useful for the design of new strategies against specific elements of the TME in order to develop better treatment approaches for GBM patients. Thus, in **Paper I**, we show that cells adapted to hypoxia (*i.e.* low oxygen) make use of the surrounding lipids for energy production and secrete proteins that signal for the quick assembly of more

blood vessels in an attempt to mitigate nutrient deprivation and support further tumor growth. Interestingly, these hypoxic cells were also seen to attract macrophages (*i.e.* a type of immune cells) into the tumor. Knowing that cancer cells do all things possible to evade the immune system, can you reason why would they attract a surveillance cell into the tumor? While you think more about it, in **Paper II** we describe and apply a method denominated the TS-MAP, which is a method created to identify the proteins at the surface of cancer cells. This approach helps comprehend which proteins are displayed at the surface of cancer cells, similar to identifying a person by a set of physical characteristics they present, to build a tumoral portrait. More interestingly, the method also allows the identification of proteins capable of entering the cell. Uptaken proteins are good "Trojan horses", being used for the targeted delivery of drug molecules directly to the cancer cell. This strategy was further explored in the production of **Paper III**, where we applied the TS-MAP methodology to uncover the landscape of surface and internalizing proteins of hypoxia-adapted cancer cells. We then applied a treatment approach known as antibody-drug conjugate (ADC), which uses an antibody loaded with a cell-killing compound to bind to some of those surface proteins, killing the targeted cell once the protein-ADC complex is internalized. Lastly, going back to the question from the first paper, **Paper IV** presents an ongoing study showing that macrophages get attracted to the hypoxic niche in a trick to get them stuffed with lipids. Consequently, the stuffed macrophages lose their ability to work properly and turn into tumor-promoting cells.

In conclusion, this thesis contributes to the current knowledge of GBM and brings us a step closer to unraveling the complexities of TME. This work also provides the experimental assessment of valuable tools, such as TS-MAP, for the identification of potential therapeutic targets in hopes of providing strategies to assist the future development of better treatment options for GBM patients.

# Sumário popular em Português

Câncer é uma doença que afeta milhões de pessoas ao redor do mundo. A doença surge quando células anormais do corpo começam a crescer e se espalhar incontrolavelmente, atacando tecidos saudáveis ao longo do caminho. Nesta tese, o foco é dado à investigação do glioblastoma (GBM), um tipo agressivo de câncer cerebral, ainda incurável e diagnosticado majoritariamente em adultos. Os estudos inclusos foram desenvolvidos para ajudar a aumentar o conhecimento atual sobre este tipo de tumor e para apresentar potenciais ferramentas auxiliares no combate a esta enfermidade.

Utilizando uma metáfora de comunidade, nós podemos considerar as células cerebrais saudáveis como os residentes de uma cidade movimentada. Comparativamente, o surgimento de um tumor GBM representaria a chegada de um grupo de ladrões agressivos que se expande rapidamente e que habilmente se esquivam do sistema de vigilância. Como esperado, a presença desse grupo perturba o equilíbrio local, tornando a comunidade menos funcional ao longo do tempo. As células cancerígenas estabelecem interações caóticas entre si e com outras estruturas ao redor do tumor (*p. ex.*: vasos sanguíneos, células imunológicas e moléculas), formando um ecossistema disruptivo conhecido como o **microambiente tumoral (MAT)**. Na nossa analogia, diferentes componentes do MAT podem ser comparados com objetos essenciais que nos rodeiam, e estes contribuem diretamente para a manutenção e crescimento do câncer. Por exemplo, a **matriz extracelular (MEC)**, que é uma rede de proteínas e carboidratos, fornece o suporte estrutural que o tumor necessita, similar ao aço encontrado nas construções. Essa armação é mantida e modificada por células engenheiras conhecidas como **células estromais**, que depositam tecido conjuntivo para moldar o MAT de acordo com a demanda do tumor. As estradas representam os **vasos sanguíneos**, uma vez que estes permitem que células, metabólitos e informações transitem através dos compartimentos tumorais e saudáveis. Além disso, simbolizando as autoridades locais, as **células imunológicas** desempenham um papel vital na vigilância e na defesa contra o câncer. Dentro do MAT, estas autoridades são vistas lutando contra células cancerígenas ou sendo enganadas pelos seus truques. Por último, à medida que o volume do tumor aumenta, a taxa de formação de novos vasos sanguíneos (estradas) é incapaz de acompanhar a crescente demanda por nutrientes. Isso culmina no surgimento de zonas mal abastecidas, caracterizadas pela escassez de nutrientes essenciais e pela acidificação extracelular (baixo pH) devido ao acúmulo de resíduos. Consequentemente, uma nova característica do MAT surge: a região **Necrótica**, definida por aglomerados de células mortas que sucumbiram ao ambiente hostil.

Ao visualizar o MAT como uma vizinhança hipotética, nós podemos começar a compreender a dinâmica complexa entre os diferentes componentes do tumor. Este conhecimento é útil para a concepção de novas estratégias contra elementos específicos

do MAT, a fim de desenvolver melhores abordagens de tratamento para pacientes com GBM. Sendo assim, no **Artigo I**, nós demonstramos que as células adaptadas à hipóxia (baixo nível de oxigênio) fazem uso dos lipídios presentes como fonte de energia e estas também secretam proteínas que atuam na montagem rápida de vasos sanguíneos na tentativa de mitigar a privação de nutrientes e estimular o crescimento tumoral. Curiosamente, as células hipóxicas também atraem macrófagos (um tipo de célula do sistema imunológico) para o tumor. Sabendo que as células tumorais fazem tudo possível para escapar do sistema imunológico, você consegue adivinhar por que elas atrairiam uma célula de vigilância para dentro do tumor? Enquanto você pensa sobre isso, no **Artigo II** nós descrevemos um método denominado TS-MAP, que é um método criado para identificar as proteínas na superfície das células cancerígenas. Essa abordagem ajuda a construir um retrato falado tumoral, semelhante à identificação de uma pessoa com base nas características físicas que ela apresenta. O mais interessante é que o método também permite a identificação de proteínas capazes de adentrar a célula. Estas proteínas captadas são bons "cavalos de Tróia", sendo usadas para a entrega direcionada de medicamentos à célula cancerosa. Esta estratégia foi explorada no **Artigo III**, onde aplicamos a metodologia TS-MAP para descobrir o repertório de proteínas presentes tanto na superfície das células tumorais adaptadas à hipóxia, quanto as internalizadas pelas mesmas. Em seguida, aplicamos uma abordagem de tratamento conhecida como conjugado anticorpo-droga (CAD), que usa um composto tóxico ligado a um anticorpo que se fixa a algumas dessas proteínas de superfície, matando a célula alvo após a internalização do conjunto proteína-CAD. Por fim, voltando à pergunta do primeiro artigo, o **Artigo IV** apresenta um estudo em andamento que demonstra que macrófagos são atraídos para o nicho hipóxico como parte de um truque para enchê-los com lipídios. Consequentemente, os macrófagos estufados perdem a capacidade de funcionar adequadamente e se transformam em células que beneficiam o tumor.

Em conclusão, esta tese contribui com o conhecimento atual de GBM e nos aproxima um passo mais perto de desvendar as complexidades do MAT. Este trabalho também avalia experimentalmente algumas ferramentas promisoras, como o TS-MAP, na identificação de potenciais alvos terapêuticos, com esperança de providenciar estratégias que auxiliem no desenvolvimento futuro de melhores opções para o tratamento dos pacientes com GBM.

# Résumé populaire en Français

Le cancer est une maladie qui affecte des millions de personnes à travers le monde. Il survient lorsque des cellules anormales au sein de notre corps commencent à croître et à se répandre de manière incontrôlée, attaquant des tissus sains par la même occasion. Dans cette thèse, le focus est fait sur le glioblastome (GBM), une forme hautement agressive et actuellement incurable de tumeur cérébrale diagnostiquée chez l'adulte. Les résultats présentés ici ont été développés dans le but d'améliorer nos connaissances actuelles sur ces tumeurs et pour présenter de potentiels outils afin d'aider à combattre cette maladie.

En se basant sur un quartier métaphorique, nous pouvons considérer les cellules cérébrales saines comme les résidents natifs d'une communauté active. Ainsi, l'émergence d'une tumeur de GBM représenterait l'arrivée de cambrioleurs agressifs qui se répandrait rapidement en évitant adroitement la surveillance. Comme attendu, leur présence perturbe l'équilibre, rendant la communauté moins efficace au fil du temps. Les cellules cancéreuses établissent des communications chaotiques entre elles et avec les autres structures entourant la tumeur (par ex. des vaisseaux sanguins, des cellules immunitaires et des molécules), formant un écosystème perturbateur appelé le **microenvironnement tumoral (MET)**. Dans notre analogie, différents composants du MET peuvent être comparés à diverses infrastructures nous entourant et ainsi, ils contribuent directement au maintien du cancer et à son développement. Par exemple, la **matrice extracellulaire (MEC)**, un réseau de protéines et de glucides, fournit le soutien structurel nécessaire à la tumeur, similaire à l'acier au sein des constructions. De telles structures sont maintenues et modifiées par des cellules ingénieures connues comme les **cellules stromales**, qui produisent le tissu conjonctif pour façonner le MET selon les besoins tumoraux. Le rôle des routes est tenu par les **vaisseaux sanguins**, ces derniers permettant aux diverses cellules, aux métabolites et aux informations de transiter dans les compartiments tumoraux comme sains. De plus, tenant lieu de gardiens de la paix, les **cellules immunitaires** jouent un rôle vital de surveillance et de défense. Parmi le MET, ces gardiens sont vus tant combattant le cancer qu'étant bernés par leurs tours de passe-passe. Enfin, la tumeur grossissant toujours plus, la formation de nouveaux vaisseaux sanguins (routes) n'est plus en mesure de suivre les besoins toujours croissants en nutriments. Cet état de fait culmine avec l'émergence de zones sous-perfusées, caractérisées par la privation en nutriments essentiels et l'acidification extracellulaire (*i.e.* un pH bas) dû à l'accumulation de déchets. Par conséquent, une nouvelle région apparaît au sein du MET : la zone **nécrotique**, définie par des groupes de cellules mortes ayant succombées au milieu hostile.

En représentant le MET comme ce quartier hypothétique, nous pouvons commencer à comprendre les dynamiques complexes entre les différents composants de la tumeur.

Cette connaissance est nécessaire pour créer de nouvelles stratégies contre des éléments spécifiques du MET dans le but de développer de meilleures approches thérapeutiques pour les patients atteints de GBM. Ainsi, dans l'**article I**, nous montrons que les cellules adaptées à l'hypoxie (*i.e.* une faible oxygénation) utilisent les lipides environnants pour produire de l'énergie et sécrètent des protéines pour rapidement former plus de vaisseaux sanguins et donc contourner la privation en nutriments et soutenir la constante croissance tumorale. Il est intéressant de noter que ces cellules hypoxiques attirent aussi des macrophages (*i.e.* un type de cellules immunitaires) au sein de la tumeur. Sachant que les cellules cancéreuses font tout leur possible pour échapper au système immunitaire, pourquoi, selon vous, attireraient-elles des cellules des défenses immunitaires dans la tumeur? Pendant que vous réfléchissez plus au problème, dans l'**article II** nous décrivons et appliquons une méthode appelée TS-MAP, méthode créée pour identifier les protéines à la surface des cellules cancéreuses. Cette approche aide à comprendre quelles protéines sont exhibées à la surface des cellules cancéreuses, similaire à l'identification d'une personne à l'aide d'un jeu de caractéristiques physiques qu'elle présente, pour fabriquer un portrait tumoral. Encore plus intéressant, cette méthode permet aussi l'identification de protéines capable d'entrer dans la cellule. Ces protéines internalisées sont de bons « chevaux de Troie », pouvant être utilisées pour la transmission spécifique de médicaments aux cellules tumorales. Cette stratégie est explorée plus en détail lors de l'**article III**, où nous appliquons la méthode du TS-MAP pour découvrir la palette de protéines de surface et internalisées de cellules cancéreuses adaptées à l'hypoxie. Ensuite, nous utilisons une approche thérapeutique connue comme le conjugué anticorps-médicament (en anglais antibody-drug conjugate ou ADC), par laquelle un anticorps chargé avec un composé toxique se lie à certaines de ces protéines de surface, tuant la cellule cible une fois le complexe protéine-ADC internalisé. Enfin, revenant à la question soulevée par le premier article, l'**article IV** présente une étude en cours montrant que les macrophages sont attirés par la niche hypoxique dans le but de les gaver de lipides. Par conséquent, les macrophages gavés perdent leur capacité à fonctionner correctement et deviennent des cellules pro-tumorales.

Pour conclure, cette thèse contribue au savoir actuel sur le GBM et nous rapproche un peu plus de dénouer le nœud complexe qu'est le MET. Ces travaux fournissent également l'évaluation expérimentale d'outils précieux, tel que le TS-MAP, pour l'identification de cibles thérapeutiques potentielles dans l'espoir de fournir des stratégies soutenant le développement de meilleurs options thérapeutiques pour les patients atteints de GBM.

# Populärvetenskaplig sammanfattning på Svenska

Cancer är en sjukdom som drabbar miliontals människor över hela världen. Den uppstår när onormala celler i vår kropp börjar växa och spridas okontrollerat, medan de attackerar friska vävnader längs vägen. I denna avhandling läggs fokus på undersökningen av glioblastom (GBM), en aggressiv och än så länge obotlig typ av hjärncancer som diagnostiseras hos vuxna. Arbetet som presenteras här har utvecklats för att förbättra den aktuella kunskapen om denna typ av tumör samt för att presentera potentiella verktyg för att hjälpa i kampen mot denna malignitet.

I en grannskapsmetafor kan vi betrakta de friska hjärncellerna som de infödda invånarna i ett hektiskt samhälle. Jämförelsevis skulle uppkomsten av en GBM-tumör representera anländandet av aggressiva inbrottstjuvar som snabbt breder ut sig och skickligt undviker att bli upptäckta. Som väntat stör deras närvaro balansen, vilket gör samhället mindre funktionellt med tiden. Cancercellerna etablerar kaotiska interaktioner mellan sig själva och andra tumöromgivande strukturer (t.ex. blodkärl, immunceller och molekyler), och bildar ett störande ekosystem som kallas **tumörmikromiljön (TMM)**. I vår analogi kan olika komponenter i TMM jämföras med väsentliga saker som omger oss, och de bidrar direkt till cancers överlevnad och tillväxt. Till exempel ger den **extracellulära matrisen (ECM)**, ett nätverk av proteiner och kolhydrater, det strukturella stöd som tumören kräver, ungefär som stålet som finns i konstruktioner. Strukturen upprätthålls och modifieras av ingenjörsceller kända som **stromaceller**, som lägger bindväv för att forma TMM enligt tumörens krav. Vägarnas roll representeras av **blodkärl** eftersom dessa tillåter celler, metaboliter och information att passera in i tumören och friska vävnader. Vidare spelar **immunceller**, som symboliserar de lokala myndigheterna, en viktig roll i övervakning och försvar. Inom TMM ses dessa instanser antingen slåss mot cancerceller eller bli lurade av deras tricks. Slutligen, när tumörmassan växer sig större, kan inte bildandet av nya blodkärl (vägar) hålla jämna steg med den ständigt ökande efterfrågan på näringstillförsel. Detta kulminerar i uppkomsten av dåligt perfunderade zoner, som kännetecknas av att essentiella näringsämnen försvinner och extracellulär försurning (dvs. lågt pH) på grund av ackumulering av avfallsprodukter. Följaktligen uppträder ett nytt kännetecken av TMM: det **nekrotiska** området, som definieras av klungor av döda celler som inte har överlevt den farliga miljön.

Genom att visualisera TMM som ett hypotetiskt grannskap kan vi börja förstå den komplexa dynamiken mellan de olika komponenterna i tumören. Denna kunskap är användbar för utformningen av nya strategier mot specifika delar av TMM för att utveckla bättre behandlingsmetoder för GBM-patienter. I **Artikel I** visar vi att celler anpassade till hypoxi (dvs låg syrehalt) använder de omgivande lipiderna för energiproduktion och utsöndrar proteiner som signalerar för snabb sammansättning av fler blodkärl i ett försök att undvika näringsbrist och stödja ytterligare tumörtillväxt. Intressant nog sågs

dessa hypoxiska celler också locka makrofager (dvs en typ av immunceller) in i tumören. Med tanke på att cancerceller gör allt möjligt för att undvika immunförsvaret, kan du resonera varför de skulle vilja locka en övervakningscell in i tumören? Medan du tänker mer på det, beskriver och tillämpar vi i **Artikel II** en metod som kallas TS-MAP, som är en metod skapad för att identifiera proteinerna på ytan av cancerceller. Detta tillvägagångssätt hjälper till att förstå vilka proteiner som visas på ytan av cancerceller, på samma sätt som man identifierar en person genom olika fysiska egenskaper de presenterar, för att bygga ett tumörporträtt. Mer intressant är att metoden också tillåter identifiering av proteiner som kan komma in i cellen. Proteiner som tas in i cellen kan användas som "trojanska hästar", för riktad leverans av läkemedelsmolekyler direkt till cancercellen. Denna strategi undersöktes ytterligare i produktionen av **Artikel III**, där vi använde TS-MAP-metoden för att avslöja landskapet av yt- och internaliserande proteiner från hypoxianpassade cancerceller. Vi tillämpade sedan en behandlingsmetod som kallas antikropp-läkemedelskonjugat (ADC), som använder en antikropp laddad med cellgift för att binda till några av dessa ytproteiner och döda målcellen när protein-ADC-komplexet internaliseras. Slutligen, för att återkomma till frågan från Artikel I, presenterar **Artikel IV** en pågående studie som visar att makrofager attraheras av den hypoxiska nischen i ett trick för att få dem fyllda med lipider. Följaktligen förlorar de fyllda makrofagerna sin förmåga att fungera korrekt och förvandlas till tumörfrämjande celler.

Sammanfattningsvis bidrar denna avhandling till den nuvarande kunskapen om GBM och tar oss ett steg närmare att reda ut komplexiteten i TMM. Detta arbete tillhandahåller även den experimentella utvärderingen av värdefulla verktyg, såsom TS-MAP, för identifiering av potentiella terapeutiska mål i hopp om att kunna erbjuda strategier för att hjälpa den framtida utvecklingen av bättre behandlingsalternativ för GBM-patienter.

# List of original papers

This thesis is based on the following papers:

**I** **Extracellular lipid loading augments hypoxic paracrine signaling and promotes glioma angiogenesis and macrophage infiltration.**
Offer S, Menard JA, Enríquez Pérez J, **Gonçalves de Oliveira K**, Indira Chandran V, Johansson MC, Bång-Rudenstam A, Siesjö P, Ebbesson A, Hedenfalk I, Sundgren PC, Darabi A, Belting M.
*J Exp Clin Cancer Res* **38**, 241 (2019). https://doi.org/10.1186/s13046-019-1228-6.

**II** **Landscape of surfaceome and endocytome in human glioma is divergent and depends on cellular spatial organization.**
Governa V*****, Talbot H*****, **Gonçalves de Oliveira K***, Cerezo-Magaña M, Bång-Rudenstam A, Johansson MC, Månsson AS, Forsberg-Nilsson K, Marko-Varga G, Enríquez Pérez J, Darabi A, Malmström J, Bengzon J, Welinder C, Belting M. ***Equal contribution**.
*Proc Natl Acad Sci USA* **119**, 9 (2022). doi:10.1073/pnas.2114456119.

**III** **Decoding of the surfaceome and endocytome in primary glioblastoma cells identifies potential target antigens in the hypoxic tumor niche.**
**Gonçalves de Oliveira K**, Bång-Rudenstam A, Beyer S, Boukredine A, Talbot H, Governa V, Johansson MC, Månsson AS, Forsberg-Nilsson K, Bengzon J, Malmström J, Welinder C, Belting M.
*Acta Neuropathol. Commun.* **12**, 35 (2024). https://doi.org/10.1186/ s40478-024-01740-z.

**IV** **Lipid droplet-loaded macrophages as a targetable pro-tumorigenic immune cell entity in human glioblastoma**
Governa V, **Gonçalves de Oliveira K**,Offer S, Cerezo-Magaña M, Bång-Rudenstam A, Li J, Beyer S, Johansson MC, Månsson AS, Edvardsson C, Durmo F, Boukredine A, Gezelius E, Menard JA, Garza R, Jakobsson J, de Neergaard T, Sundgren PC, Nordenfelt P, Darabi A, Forsberg-Nilsson K, Talbot H, Bengzon J, Belting M.
*Manuscript under revision in Science Translational Medicine*

**Papers not included in this thesis:**

**Low-molecular-weight heparin adherence and effects on survival within a randomized phase III lung cancer trial (RASTEN)**
Gezelius E, Bendahl PO, **Gonçalves de Oliveira K**, Ek L, Bergman B, Sundberg J, Strandberg K, Krämer R, Belting M.
*Eur J Cancer* **118**, 82-90 (2019). doi: 10.1016/j.ejca.2019.06.015.

**Circulating Levels of the Cardiovascular Biomarkers ST2 and Adrenomedullin Predict Outcome within a Randomized Phase III Lung Cancer Trial (RASTEN)**
Gezelius E, Bendahl PO, Gallo W, **Gonçalves de Oliveira K**, Ek L, Bergman B, Sundberg J, Melander O, Belting M.
*Cancers* **14**, no. 5, 1307 (2022). https://doi.org/10.3390/cancers14051307

**Global extracellular vesicle proteomic signature defines U87-MG glioma cell hypoxic status with potential implications for non-invasive diagnostics.**
Indira Chandran V, Welinder C, **Gonçalves de Oliveira K**, Cerezo-Magaña M, Månsson AS, Johansson MC, Marko-Varga G, Belting M.
*J Neurooncol.* **144**, no. 3, 477-488. (2019). doi: 10.1007/s11060-019-03262-4.

## Main Abbreviations

| | |
|---|---|
| ADC | Antibody-Drug Conjugate |
| AKG | Alpha-Ketoglutarate |
| BBB | Blood-Brain Barrier |
| BMDM | Bone Marrow-Derived Macrophage |
| CAR-T Cell | Chimeric Antigen Receptor T Cell |
| CNS | Central Nervous System |
| DEG | Differentially Expressed Gene |
| DGAT1 | Acyl-CoA Diacylglycerol Acyltransferase 1 |
| EC | Endothelial Cell |
| ECM | Extracellular Matrix |
| EGFR | Epidermal Growth Factor Receptor |
| ELISA | Enzyme-linked immunosorbent assay |
| EMT | Epithelial-Mesenchymal Transition |
| EV | Extracellular Vesicle |
| FA | Fatty Acid |
| FIH | Factor Inhibiting HIF |
| FUS | Focused Ultrasound |
| GBM | Glioblastoma |
| GSC | Glioma Stem Cell |
| GSEA | Gene Set Enrichment Analysis |
| HBMEC | Human Brain Microvascular Endothelial Cell |
| HGCC | Human Glioblastoma Cell Culture |
| HIF | Hypoxia-Inducible Factor |
| HRE | Hypoxia Response Elements |

| | |
|---|---|
| ICI | Immune Checkpoint Inhibitor |
| IDH | Isocitrate Dehydrogenase |
| LCM | Laser Capture Microdissection |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LD | Lipid Droplet |
| LDHA | Lactate Dehydrogenase |
| LFQ | Label-Free Quantitation |
| LITT | Laser Interstitial Thermal Therapy |
| MCT | Monocarboxilase Transporter |
| MGMT | O-6-Methylguanine-DNA Methyltransferase |
| MMAF | Monomethyl Auristatin F |
| MRI | Magnetic Resonance Imaging |
| MSC | Mesenchymal Stem Cell |
| NF1 | Neurofibromatosis Type 1 |
| NK | Natural Killer cell |
| NOD | Non-Obese Diabetic |
| PBMC | Peripheral Blood Mononuclear Cell |
| PHD | Prolyl Hydroxylase |
| PTEN | Phosphatase and Tensin Homolog |
| PTM | Post-Translational Modification |
| RB1 | Retinoblastoma Protein |
| ROI | Region of Interest |
| ROS | Reactive Oxygen Species |
| SCID | Severe Combined ImmunoDeficiency |
| TAA | Tumor-Associated Antigen |

| | |
|---|---|
| TAF | Tumor-Associated Foam Cells |
| TAM | Tumor-Associated Macrophage |
| TERT | Telomerase Reverse Transcriptase |
| TG | Triglyceride |
| TGFB | Transforming Growth Factor Beta |
| TME | Tumor Microenvironment |
| TMZ | Temozolomide |
| TP53 | Tumor Protein p53 |
| TSA | Tumor Specific Antigen |
| TS-MAP | Tumor Surfaceome Mapping |
| VEGFA | Vascular Endothelial Growth Factor A |
| VHL | Von Hippel-Lindau |
| WHO | World Health Organization |

# Preface

Even though I was not aware of it at the time, I believe my academic interest emerged thanks to the second-hand bookshop visits I had with my mom, and was further fuelled by my sister's collection of Agatha Christie's books. It fascinated me how all those detectives in the stories could connect several points and notice patterns that no one else could see. Trying to understand how cancers happen, to me, feels like trying to solve a detective's mystery. You are detective Poirot, being presented with several different pieces of information, and cancer is possibly the butler, hiding in plain sight. Which hypotheses are correct? What are the patterns and routes the killer cell utilizes to escape? How to catch it and stop it from causing harm? The more I learned about how everything is connected (gene regulation, transcriptional networks, protein feedback loops) and complex, the more I dug into the research and the bigger the mystery became. Instead of one criminal weapon, cancer has dozens, all utilized at the same time for maximum efficiency: therapy evasion, stem cells, metastasis. The list goes on. Instead of a mustache for disguise, it uses thousands of proteins to pass by surveillance as a normal cell. There was always a next challenge, and I knew I had to jump in and see what I could find. While my motivation to start came from pure curiosity, my persistence to stay within the academic field was fed by my naïve rage over this devastating disease. If I could only do something to help my fellow researchers or any patient in the future, I knew it would be time well spent.

> "The impossible could not have happened, therefore the impossible must be possible in spite of appearances."
> — *Hercule Poirot*

This thesis is not meant just for academics (though I hope you will enjoy it if you are in academia as well). It is meant for anyone curious, who enjoys biology, and especially, for anyone starting in the field of cancer/GBM research. I hope this thesis gives you a theoretical base to build on, and that it helps you demystify some complex processes explained simply (as much as I could). I dare say that I hope this piece of work gives you the "AHA!" moment after finally understanding a scary concept (these are my favorite moments, professionally).

I can't wait to share it with you.

Kelin Gonçalves de Oliveira

March 2024

# Introduction

The brain, a complex and remarkable organ, is the command center of our existence. Through a vast network of interconnected neurons, it controls and coordinates a multitude of elaborate processes ranging from crucial bodily functions to cognition. Thus, the emergence of disorders that disrupt the delicate balance within the brain, such as a tumor, can result in profound and far-reaching consequences to a person's life. To improve the quality of life for patients, the design of impactful interventions is paramount, and the comprehension of the underlying mechanisms ruling the given disorder is the base of this process.

In this thesis, I invite you into my academic journey exploring one of the most common brain tumors diagnosed in adults and how specific aspects of its microenvironment influence tumor biology and the landscape of targetable proteins. The work compiled in this thesis was performed with the aim of better understanding how different players of stress adaptation (*e.g.* lipids and hypoxia) contribute to tumor aggressiveness and to explore potential therapeutic targets.

# Glioblastoma

## Epidemiology, Classification and Etiology

Glioblastomas (GBMs) are highly malignant tumors that arise in the brain *de novo*, meaning these develop without previous evidence of a lower malignancy lesion [1, 2]. This type of tumor, regarded as primary GBM, accounts for up to half of all primary malignant brain tumors diagnosed in adults (Figure 1) [3] and presents with an age-adjusted incidence of 3.2 cases per 100 000 people, according to US data, occurring more frequently in caucasian men of approximately 65 years old [2, 4, 5].

According to the fifth edition of the World Health Organization (WHO) classification of central nervous system (CNS) tumors released in 2021 [6], GBMs are diffuse grade IV astrocytomas that mandatorily present with wildtype Isocitrate Dehydrogenase (IDH) and histone 3 genes, and display at least one of the following histological or molecular alterations:

- Microvascular proliferation;
- Necrosis;
- EGFR amplification;
- TERT promoter mutation; or
- Gain of chromosome 7 combined with loss of chromosome 10.

In addition to the molecular features, GBMs usually present with retained nuclear expression of ATRX, as well as CDKN2A/B homozygous deletion on 9p21 [6]. Despite not being of diagnostic importance, the methylation status of the O-6-Methylguanine-DNA Methyltransferase (MGMT) promoter is a crucial molecular characteristic routinely profiled alongside diagnostic markers in potential GBM biopsies due to its powerful prognostic value [3] (see section **Histology and genetic/epigenetic markers** for more information on MGMT promoter status).

In the 2021 WHO classification, gliomas previously classified as low-grade tumors (those lacking microvascular proliferation or necrosis, for example) can now be regarded as GBMs, provided they fit the molecular profile. This change was due to the observation that histologically lower-grade gliomas with wildtype IDH and diffuse pattern behaved clinically as GBM (denominated molecular GBMs), and presented similar overall survival [3]. However, while the histological GBM usually presents with contrast-enhanced and hyperintense regions with necrotic patterns in Magnetic Resonance Imaging (MRI) (see section **Symptoms and Diagnosis**), molecular GBMs might differ. In a study by Guo *et al.*, a lower number of patients with molecular GBM presented with contrast enhancement and necrosis in MRI imaging [7], underscoring the importance of combining histology, imaging, and molecular features for correct diagnosis of highly heterogeneous tumors.

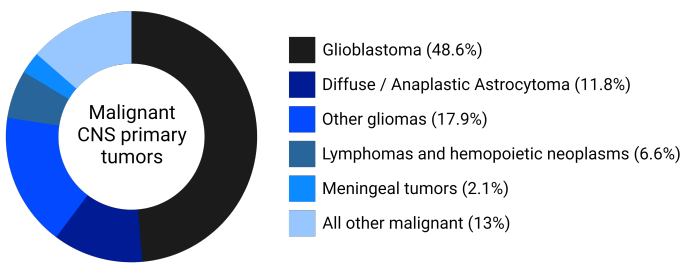


**Figure 1. Diagnosis frequency amongst malignant CNS tumors**.
Simplified distribution of CNS tumors between 2013 and 2017 according to data collected by the Central Brain Tumor Registry of the United States. Modified from Miller *et al.*, 2021 [4].

As the classification suggests, astrocytes (*i.e.* stellate cells that regulate the blood-brain barrier (BBB) and provide synaptic support between neurons) comprise the most com-

mon origin cells that give rise to this tumor [8]. However, more recent studies suggest that GBMs can also originate from the pluripotent neural stem cells or oligodendrocytes precursor cells [9–11] (Figure 2). Regarding tumor onset, GBMs commonly develop within the lobes of the supratentorial area of the brain (Figure 2) and are rarely seen having the cerebellum or brain stem as primary sites [5]. To date, the primary tumor onset location seems to be unpredictable, and patients face a similar median overall survival of mere 12-15 months, mostly driven by its invasive nature [2, 8]. Moreover, GBM cell populations develop resistance to therapeutic interventions, and tumor recurrence inevitably occurs in around 90% of cases within 7-12 months after diagnosis [12, 13]. Only around 6% of GBM patients are able to reach 5 years of survival, and little has changed regarding patient survival since the establishment of the Stupp protocol in 2005 (see section **Treatment Modalities**) which has become the current standard of care [14].

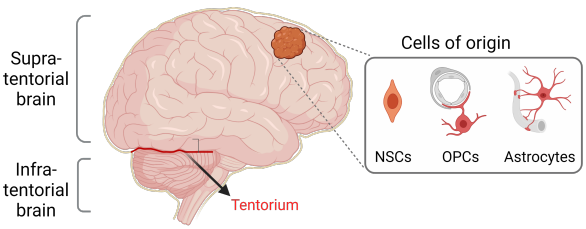


**Figure 2. GBM origin**
GBM's onset site and possible cells of origin. NSCs: Neural Stem Cells; OPCs: Oligodendrocyte Precursor Cells. Made with BioRender.

The risk factors driving GBM onset are not yet fully understood. Age above 50 years, male gender [15], and exposure to ionizing irradiation [2, 16] are the only known risk factors associated with the development of GBM. Additionally, patients presenting hereditary cancer syndromes such as neurofibromatosis [2], Lynch syndrome [17], Li-Fraumeni syndrome, and Turcot syndrome [18] are believed to also display a predisposition to GBM emergence since some of the mutated genes (*e.g.* NF1 and TP53) implicated on the condition's pathogenesis are known contributors of tumorigenesis [19]. Regarding lifestyle, obesity has been suggested to be a risk factor for GBM onset by some studies [5, 20]. However, recent meta-analytical works found no clear evidence for such association [21, 22], and dietary patterns showed little to no effect on glioma risk according to a large-scale cohort study with approximately 1.2 million participants [23]. During a systematic assessment of several published hypothesized glioma risk factors, Howel and collaborators [24] observed that increased alcohol consumption and extreme childhood obesity (Odds Ratios 4.42 and 1.11, respectively) were lifestyle factors significantly associated with glioma risk. Interestingly, the second highest risk predictor in this study was longer telomere length (Odds Ratio = 4.09), which was also observed by other studies [25] and has been correlated with increased

risk of adolescent-onset ependymoma [26]. Overall, further research is needed to better comprehend the real influence of different risks of GBM. The onset of such malignancy may, to a large degree, be a stochastic event resulting from evolutionary processes occurring in a large number of cells.

## Symptoms and Diagnosis

Symptoms of GBM widely vary amongst patients and depend on initial tumor location. Since GBM is a fast-growing cancer, initial symptoms are commonly associated with the compression of anatomical structures from both the progressive enlargement of the tumor mass and the presence of surrounding vasogenic edema [2]. Some of the main symptoms are new onset headaches, nausea, neurological or cognitive deficits, behavior changes, impaired muscle tone, loss of balance, mild to severe seizures, vision impairment, and speech difficulties [2, 5].

During the clinical investigation, a possible GBM diagnosis is further supported by sophisticated imaging methods such as Computed Tomography (CT or CTA scan), MRI, and Positron Emission Tomography (PET) scans. Due to its superior soft tissue contrast and ability to detect subtle morphological differences between tumor and normal brain tissue, MRI is the preferred diagnostic modality for GBM. In MRI, the brain images are acquired before and after the patient receives a gadolinium contrast solution, which shows the tumor as a bright region after contrast uptake, thus, indicating the presence of high-grade astrocytoma cells. Therefore, in contrast-positive T1-weighted images, the GBM lesion appears as an enhanced mass surrounding central necrosis, and under T2-weighted FLAIR imaging, it presents as a hyperintense event of tumor infiltration and edema (Figure 3A-B) [27].

Additionally, CT and PET scans may be used to detect fluids (*e.g.* hemorrhage or vasogenic edema) and assess metabolic activity, respectively. With the combined use of MRI, CT, and PET scans, suspected GBMs can be initially diagnosed but, despite the high diagnostic sensitivity of the aforementioned imaging techniques, the biopsied tissue is further sent to confirmatory histological and molecular assessments for accurate tumor subtyping, which helps clinicians to design the most appropriate treatment plan to maximize patient outcomes.

## Histology and genetic/epigenetic markers

In GBM, the main histological markers are a high number of mitotic figures, increased cellular density, microvascular proliferation with vascular thrombosis, nuclear atypia, and a large area of pseudopalisading necrosis (Figure 3C). Moreover, unlike low-grade

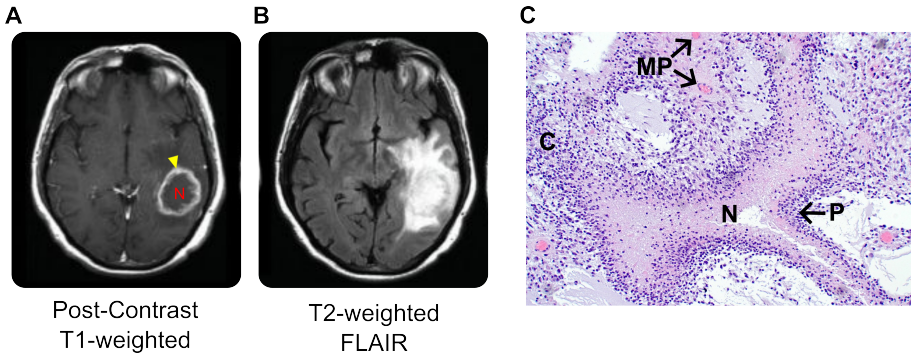


**Figure 3. Classical GBM features.**
**A)** Axial T1-weighted post-contrast image shows tumor rim (yellow arrowhead) surrounding necrosis (red N). **B)** Axial T2-weighted FLAIR image: bright area indicates region edema and/or tumor-infiltrating cells. Reprint with permission from Oxford University Press. (Naeini *et al.*, 2013.) [28] (Modified). **C)** H&E staining of GBM tissue. MP: Microvascular Proliferation; C: Dense celularity; N: Necrosis; P: Pseudopalisating cells. Reprint with permission from PathologyOutlines.com, courtesy of Bharat Ramlal, M.D. [29] (modified).

gliomas, GBM presents with undefined tumor borders due to a high degree of tumor-cell infiltration into the surrounding healthy tissue [30, 31].

Similar to histological markers, genetic markers are also important tools to enhance glioma diagnosis and are predictors of treatment response and prognosis. One example of it is the mutational status of the IDH 1/2 genes. Clinically, the detection of wildtype IDH (gene or protein) is imperative for GBM diagnosis, since mutated IDH indicates the diagnosis of lower-grade gliomas or grade IV astrocytoma [6]. In its wildtype form, the IDH 1/2 enzymes display high affinity for both isocitrate and NADP+ (Figure 4A1-2), generating alpha-ketoglutarate (AKG) and NADPH as products [32]. Both products are directed for the quenching of reactive oxygen species (ROS), and AKG also plays an important role as an intermediate of the citric acid cycle. Naturally, the conversion of AKG into either D or L 2-hydroxyglutarate enantiomers does not occur abundantly in healthy cells (Figure 4A3), being quickly converted back into AKG [33]. The produced AKG assists gene transcription regulation by serving as an indispensable co-factor for de-methylating processes (Figure 4A4) and also assists redox balance [32, 34].

On the other hand, the missense mutation of the IDH gene, seen among lower-grade tumors, culminates into an amino acid substitution within its active site, causing affinity loss to isocitrate and leading it to catalyze its previous products, AKG and NADPH (Figure 4B1-2) [32]. The abnormal consumption of NADPH leads to oxidative stress (Figure 4B2), and oncometabolite production (Figure 4B3), which disrupts the inhibition of DNA and histones de-methylation, causing DNA hypermethylation (Figure 4B4). Moreover, D-2-hydroxyglutarate also disrupts the electron chain, DNA repair

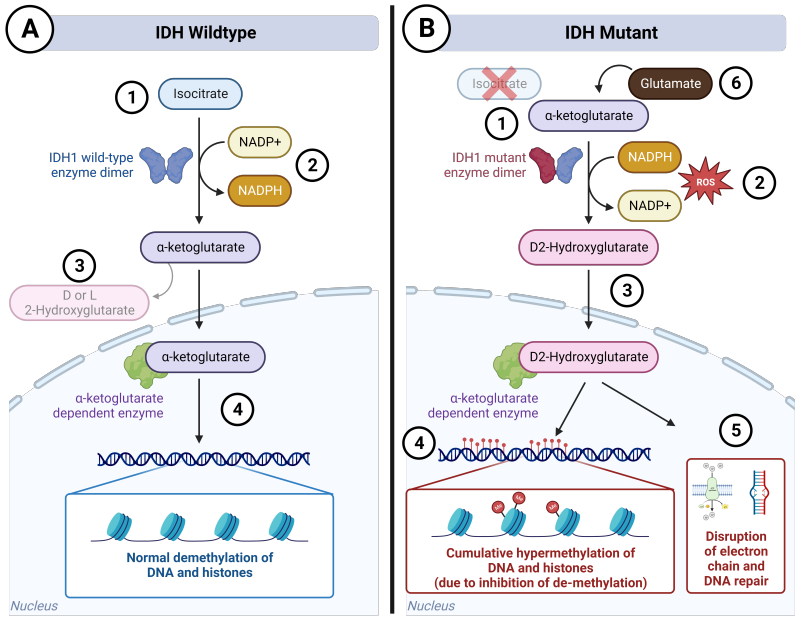


**Figure 4. The impact of IDH status in cells.**
**A)** Schematics of the functioning of wildtype IDH. (1) Isocitrate is the main IDH substrate and, (2) by transferring hydrogen to NADP+, it produces NADPH and alpha-ketoglutarate (AKG). (3) Conversion of AKG to 2-hydroxyglutarate (2-HG) is faint in healthy IDHwt cells. (4) AKG then exerts its function as a metabolic/genetic regulator. **B)** Schematics of the functioning of mutated IDH. (1) Instead of Isocitrate, the available AKG is now the preferred substrate used by the mutated enzyme. (2) Depletion of NADPH impairs the redox balance of the cell, as the available hydrogen is now being used for (3) the production of 2-HG, which (4) hinders the de-methylation of DNA and histones, and (5) disrupts energy production and DNA repair. Lastly, due to consumption of AKG for the citric acid cycle, (6) the cell uptakes glutamate as a metabolic substitute. Made with BioRender.

mechanisms (Figure 4B5), and transaminases activity [32, 35]. AKG absence causes cells to be dependent on glutamate as the substrate for the compensation of metabolic imbalances (Figure 4B6). Overall, the metabolic and epigenetic changes render IDH-mutated cells prone to cell cycle arrest, reduced proliferation, and sensitivity to disturbances. Thus, treatment with glutaminase inhibition, chemotherapy, and radiotherapy, confer better prognosis to IDH-mutated patients [32, 35, 36].

Although the IDH gene is kept unaltered, primary GBMs are known to alter more than 600 genes converging towards constant activation of proliferative pathways, as well as evasion of anti-tumoral processes such as senescence, cell cycle checkpoints, and death, highlighting the aggressiveness of the disease [36–38]. Some of the commonly seen alterations contributing to aberrant signaling are EGFR gene amplification (or over-activating EGFR mutation), and mutations in key proliferation genes such as

gain of function PIK3CA and PIK3R1 alterations [39] as ways to sustain continuous cell proliferation and tumor growth. Moreover, tumoral cells also present mutations in tumor-suppressive genes such as PTEN [40], TP53 [5], TERT [5], RB1 [41], and NF1 [42]. In addition to mutations, chromosomal aberrations are a commonly seen feature of GBMs and include amplification of chromosomes 4, 7, and 12, leading to overexpression of pro-tumorigenic genes such as PDGFRA (Chr. 4), EFGR and MET (Chr. 7), CDK4 and MDM2 (Chr. 12), as well as the silencing of tumor-suppressors via chromosomal deletion or loss of heterozygosity in genes such as PTEN in chromosome 10, allowing cells to mitigate the inhibition of proliferative axes (upregulating the PI3K/AKT/mTOR proliferative pathway, in the case of PTEN/Chr10 deletion and EGFR amplification) [36, 37, 43].

Explorations on how the mutational landscape of GBM evolves between primary and recurrent tumors have identified few consensus patterns, possibly due to the highly personalized genetic roots of GBM [44]. Recurring GBMs were reported to retain some of their primary features such as lack of IDH mutation and retention of TERT promoter mutation [45–47]. Still, most patients presented a branched evolutionary pattern. A rise in the prevalence of driver gene mutations, *e.g.* EGFR (19% / 30% primary/recurrent) and CDKN2A (3% / 18%), have been observed [48]. Moreover, genetic/epigenetic alterations were seen to influence the fate of neural stem cells within the subventricular zone towards pro-tumorigenic phenotypes in recurrent tumors [44]. Interestingly, the treatment of IDH-wildtype primary GBMs with a chemotherapy regime was not associated with the emergence of hypermutated recurrences, and cells from recurrent tumors seemed to retain similar genetic and epigenetic phenotypes to their primary tissue [46, 47]. Thus, the heterogeneity of GBM is also represented in recurrent tumors, but the elucidation of the evolutionary routes of recurring cells depends on the design of more comprehensive multifaceted studies covering transcriptional, spatial, and (epi)genetic information [45, 46, 49].

Both in primary and recurrent settings, GBM's genetic landscape is highly influenced by epigenetic modulations [50]. Notably, the DNA methylation pattern of the MGMT gene promoter in GBM is an epigenetic marker of great importance, acting as an effective predictive marker for response to treatment. Specifically, hypermethylation of the MGMT promoter region was shown to be associated with better patient outcomes ([51]) as it leads to the downregulation of its gene product, a DNA-repairing enzyme. Consequently, the reduced production of such enzyme reduces tumor-cell recovery after treatment with alkylating chemotherapy (*e.g.* temozolomide) by inhibiting the tumor cells from correcting drug-induced DNA damage, leading to more death of malignant cells, and ultimately resulting in increased patient survival rates [5].

## GBM transcriptional subtypes

Atop genetic/epigenetic alterations, GBM also exhibits distinct transcriptional subtypes, which were initially identified through analysis of gene expression patterns of several GBM samples. The currently most utilized GBM subtype classification was defined by Verhaak and collaborators in 2010 upon unsupervised analysis of 202 newly diagnosed GBMs [52]. These subtypes, named proneural, mesenchymal, classical, and neural, reflect diverse biological processes and cellular functions within the tumor milieu and were associated with variations in treatment response, patient survival, and disease progression. Upon further revision of the GBM subtypes through the lens of single cells sequencing [53] and deep-learning algorithms [54], the neural subtype was ruled out as a true tumor-intrinsic transcriptional signature, as it was discovered to have originated from sample contamination with non-tumoral cells.

Amongst the remaining three subtypes, tumor cells classified as mesenchymal presented with the most aggressive profile characterized by expression of mesenchymal markers like CHI3L1, MET, MERKT, and CD44, elevated activation of the MAPK proliferative pathway, increased presence of inflammation markers (fibronectin and COX-2), and increased expression of endothelial markers like CD31 and VEGFR-2 when compared to the other subtypes. Additionally, mesenchymal GBM cells were seen to exhibit a lower degree of tuberin, which regulates mTOR, a crucial pathway in cancer biology that governs protein synthesis for cell proliferation, survival, and other pro-tumorigenic processes [53], as well as frequent co-deletion or inactivating mutations of tumor-suppressor genes like NF1 and PTEN [42, 52]. High expression of genes associated with the tumor necrosis factor pathway is also present, such as TNFRSF1A and TRADD. As a consequence, mesenchymal GBM is associated with a worse prognosis, therapeutic resistance, higher tumor necrosis, and a high degree of macrophage infiltration [52, 55, 56].

Cells from the proneural subtype were also found to upregulate the mTOR pathway but through 4EBP1 inhibition [37]. Moreover, through the high expression of oligodendrocytic development genes like PDGFRA, NKX2-2, and OLIG2, the proneural subtype was seen to be enriched for alterations in genes associated with neural development and differentiation. PDGFRA is associated with sustained activation of the Ras/MAPK signaling pathway and, in proneural tumors, is frequently amplified [52]. The homeodomain protein NK2 homeobox 2 (NKX2-2) and OLIG2 genes, also observed to be upregulated in this subtype, are crucial transcription factors for the control of cell-fate specification and differentiation. Both genes regulate oligodendrocyte differentiation and, when co-activated, were found to induce the expression of myelin-related genes [57]. OLIG2 also plays a role in sustaining replication in early development through the induction of chromatin remodeling events, and it counter-acts astrocytic maturation

[58]. Of all the subtypes, patients with proneural GBM displayed a trend toward longer survival, even though this subtype was reported to lack immune infiltration [52, 56].

Lastly, the classical GBM subtype resembles the typical GBM morphology, exhibiting intermediate characteristics between mesenchymal and proneural subtypes. Due to the amplification of chromosome 7, this subtype presents with overexpression of the EGFR gene [52, 59]. Moreover, deletion of the CDKN2A gene, *i.e.* an important regulator of cell growth [60], was also frequently observed in classical GBM, resulting in the inactivation of the RB pathway via RB1, CDk4, and CCDN2, as well as redistribution of polyunsaturated fatty acids into lipid compartments to confer GBM cell protection against ROS [43, 61]. Other aberrations such as chromosome 10 loss and over-activation of the sonic-hedgehog and notch signaling pathways are also characteristics of classical GBM [43].

Subtyping GBM tumors according to their transcriptional profile is a complex task, and different authors have classified them differently throughout the years. Previously to Verhhak, Phillips and collaborators first described the GBM subtypes in 2006 as proneural, proliferative, and mesenchymal, in which the proliferative subtype was enriched for neuronal stem cells markers [36, 62]. Later, in 2013, Brennan clustered GBM cells into 6 groups (M1-M6), where M1 and M2 clusters were enriched for mesenchymal signature, M3 and M4 resembled Verhaak's classical subtype, M5 was classified as the glioma-CpGs island methylator phenotype, and M6 was enriched for hypomethylated proneural cells [37]. In summary, regardless of the preferred way to subdivide tumor cells (by transcriptional subtypes, genomic and histological markers), GBM displays a remarkable degree of tumor heterogeneity.

Rather than one GBM cell subtype, deconvolution strategies have demonstrated that a tumor is composed of a mosaic of cell subtypes with different cell-of-origin and gene signature contributions [63, 64]. The presence of multiple intermediary cell states is a strong illustration of GBM plasticity, which is able to maintain primed cells ready to switch transcriptomic states based on the activation of nonhierarchical differentiation programs [56]. In relapsed GBMs, approximately two-thirds of patients exhibited different transcriptional subtypes compared to their matched primary tissue [45]. The pluripotency of GBM cells complicates the development of new therapeutic strategies as a one-size-fits-all approach often falls short when faced with the myriad variations found in this type of cancer, requiring therapeutical combinations for targeting of multiple cellular states [65].

# The Tumor Microenvironment

Tumor microenvironment (TME) is the term used to refer to the complex milieu of solid tumors. In GBM, the TME comprises a dynamic network of non-malignant cells such as neurons, glial cells (*e.g.* oligodendrocytes, pericytes, astrocytes), vascular endothelial cells (ECs), infiltrating and resident immune cells (*e.g.* macrophages, microglia, lymphocytes, neutrophils), soluble factors (*e.g.* cytokines, growth factors, enzymes), and vesicles (*e.g.* exosomes, oncosomes, apoptotic bodies), all immersed in a modified extracellular matrix (ECM) [13, 66]. Additionally, the TME of aggressive tumors, as is the case for GBM, presents active stress factors such as low oxygen tension and acidosis (*i.e.* low pH) due to poor tissue perfusion and accumulation of waste materials. These trigger adaptive responses leading to the selection of resistant tumor clones that evade treatment and further contribute to tumor aggressiveness, recurrence, and poor patient prognosis. Rather than a passive tumor feature, the TME is reprogrammed and continuously modulated by tumor cells to actively promote tumor growth, spread of malignant cells, resistance to therapies, and transformation of the surrounding normal tissue towards a pro-tumorigenic environment [13].

## Cellular Component and Extracellular Matrix Remodeling

The TME exhibits distinct cell types playing crucial roles in tumor progression. Parenchymal cells such as pericytes, astrocytes, and neurons contribute to tumor structure through intercellular communication. Pericytes, *i.e.* mural cells enveloping the vascular endothelium, stabilize the capillary network and establish the BBB, *i.e.* a highly selective vascular network of tightly packed ECs that gatekeep the parenchyma [66, 67]. Their absence around the newly formed blood vessels is associated with BBB disruptions and the leaky vasculature seen in GBM tumors. Astrocytes interact with pericytes, participating in BBB maintenance, and mediating their communication with neural cells for metabolic, synaptic, and microglial regulation. Furthermore, tumoral cells also mediate communication with neuronal cells via neuron-to-glioma synapses and paracrine signaling, contributing to phenotypic adaptations of tumor cells, TME, and its components [56, 68].

Mesenchymal stem cells (MSCs), recruited to the TME by tumor-derived signals (*e.g.* TGFB1, neurotrophin 3, and CXCL12), are stromal cells with self-renewal and mul-

tilineage differentiation capabilities [69]. While MSCs influence the TME, their role is not yet fully elucidated, and they carry context-dependent roles [69]. Studies by Breznik *et al.* [70] demonstrating the role of MSC-released metalloproteinase in facilitating ECM remodeling and cell migration showed that they may support tumor progression, while the studies by Ho *et al.* [71] indicated that MSCs can lean towards an anti-tumorigenic state as evidenced by the angiogenic inhibition observed after tumor cell treatment with bone marrow-derived MSCs.

Differently from MSCs, glioma stem cells (GSC) present tumor-initiating abilities but these cells also present self-renewal potential. Characterized by the expression of surface markers (*e.g.* CD133, SOX2, Nestin, CD44) GSCs are further subdivided into proneural or mesenchymal cells based on their transcriptional signature. The proneural GSCs, located at the perivascular tumor niche display a proliferative behavior while mesenchymal GSCs are commonly found within the hypoxic niche as quiescent cells [55].

The GBM microenvironment is further populated by immune cells, which can constitute up to 50% of the bulk's cellularity and consist of microglia and bone marrow-derived macrophages (BMDMs) (both populations are generally referred to as tumor-associated macrophages (TAMs)), together with dendritic cells, neutrophils, natural killer (NK) cells, and T cells [38]. Microglia are the resident scavenging cells of the CNS and can be polarized towards a tumor-supportive phenotype by factors secreted by GSCs and stromal cells. Leukocytes, such as neutrophils, and cells of lymphoid lineage (NK and T cells), are poorly represented within the TME. While neutrophils were seen to be somewhat present in GBMs, these were mostly immunosuppressive as well, and NK cells from IDH wildtype tumors were predominantly in their immature $CD16^-$ state, instead of the $CD16^+$ cytotoxic form [13, 66]. Regarding lymphocyte status, GBM is considered a cold tumor due to its low T cell infiltration and the TME contributions towards their inactivation. One of the factors contributing to the tumor's coldness is the density of the ECM and the limited vascularization within the TME, which physically restricts T cell trafficking and hinders their ability to access tumor cells. Also, the immunosuppressive TAMs help orchestrate immune escape via the production of anti-inflammatory cytokines (IL-4 and IL-10), and activation of immune checkpoint proteins (PD-L1 and CD39) [13, 72]. Tumor cells further contribute to the inhibition of immune cell activity by secretion of soluble mediators such as TGFB, prostaglandin E2, and IL-10 [73]. Moreover, the low tumor mutational burden of GBM generally results in a limited repertoire of tumor-specific antigens for T cell recognition, compromising the ability of T cells to mount a robust immune response [13].

Within the GBM TME, TAMs release pro-angiogenic factors (*e.g.* VEGF), and reinforce proliferative pathways such as EGFR. These pro-tumorigenic signals promote the sprouting of blood vessels (angiogenesis) and evoke ECM remodeling to facilitate

tumor cell invasion, migration, and resistance to therapy [13].

Similarly to TAMs, dendritic cells are also antigen-presenting cells within the GBM TME and exhibit an immunosuppressive phenotype. They are important meningeal mediators of the MHC-I-based antigen presentation process and are crucial for T-cell priming and activation. Nevertheless, the lack of parenchymal lymphoid vessels hinders the presentation of tumoral antigens by dendritic cells, which instead occurs via the MHC-II-based presentation, mediated by the TAMs within the tissue. BMDMs originate from peripheral blood monocytes that have egressed the bone marrow and infiltrated the tumor milieu, differentiating into macrophages. TAMs can exhibit phagocytic activity towards apoptotic and necrotic GBM cells, recognizing "eat-me" signals such as exposure of calreticulin and phosphatidylserine on their outer plasma membrane [74]. This clearance of dead and dying tumor cells can limit tumor growth and promote an anti-tumoral immune response. However, antigen presentation of a macrophage subpopulation is greatly impaired by STAT3 tumor overexpression that inhibits TAMs, decreasing the chances of MHC-mediated immune activation against tumor cells [72]. Moreover, the phagocytic capacity of the tumor-promoting TAMs is believed to have at least two major roles in favoring GBM: first, the scavenging of dead tumor cells by TAMs could help reduce the availability of tumoral antigens exposed to antigen-presenting cells; and second, as proposed in **Paper IV** (unpublished), the phagocytosis of lipids has the potential of stuffing TAMs, turning them into tumor-associated foam cells (TAFs). TAFs are characterized by the abundant presence of intracellular lipids, and it is suggested that these lipid-rich cells could support tumor development by immunosuppression and increased angiogenesis. In **Papers I** and **IV**, we explore the role of lipids in promoting a pro-tumorigenic TAM phenotype. Rather than eradicating all TAM populations, therapeutical efforts could benefit from enriching the tumor bulk with anti-tumoral TAMs while destabilizing pro-tumoral TAMs and TAFs.

Lastly, the ECM is a complex network of proteins, glycosaminoglycans, and polysaccharides that play a critical role in the GBM microenvironment [75]. It provides structural support for the tumor mass, regulates cellular signaling pathways within GBM cells, and influences their migration. GBM cells secrete various enzymes (*e.g.* metalloproteinases) that degrade and remodel the ECM, creating migratory channels and promoting angiogenesis by aiding the formation of new blood vessels. Additionally, the composition and stiffness of the ECM can influence GSC behavior. Stiffer ECM environments have been shown to promote the self-renewal and the tumorigenic potential of GSCs. Conversely, softer ECM environments may favor differentiation and reduce tumorigenicity [76].

## Hypoxia

Deficient oxygen availability, or hypoxia, is a recognized hallmark of GBM and arises from the combination of rapidly expanding tumor mass which exceeds the capacity of its vasculature, thrombotic events due to EC loss, and remodeling of cancer morphology causing compression of vascular structures. These factors ultimately culminate in insufficient oxygen and nutrient supply to the tissue [77]. GBMs present with a gradient of $O_2$ tension established based on the proximity of the tumor cells to the blood vessels. Previous studies have shown that hypoxic areas of the embryonal or tumoral brain reach around 1% of oxygen (approximately 7.2 mmHg). However, the oxygen tension gradient of GBM is dynamic and can vary from 0.1 to 2.5% $O_2$ (severe to moderate hypoxia, respectively) depending on the tumoral region measured [78, 79]. Thus, cells located near vasculature, the perivascular niche, are well-perfused and receive near-normal oxygen supply, while those further away from it experience a progressive decline in oxygen tension at the pseudopalisading necrosis region. Hypoxia within the TME triggers a complex cascade of cellular responses largely mediated by the transcription factor family named hypoxia-inducible factors (HIFs) 1-3 alpha [80]. While HIF 3 is less studied and suspected of negative regulation of the other family members, HIFs 1 and 2 alpha are well-known proteins that act as intracellular oxygen sensors and induce transient changes in the expression of numerous genes when $O_2$ levels are low, promoting cell migration, survival, and other pro-tumorigenic processes [81]. Importantly, in an attempt to increase oxygen delivery, one of the main HIF-driven responses is the promotion of angiogenesis. However, the newly formed vessels are often dysfunctional and leaky, further perpetuating the hypoxic state [80].

Structurally, HIFs are heterodimeric proteins, composed of an alpha and beta subunit. While the beta subunit (ARNT) is constitutive, the alpha subunit is oxygen-sensitive and, under normoxic conditions, undergoes hydroxylation by prolyl hydroxylases (PHDs) and asparaginyl hydroxylases (factor inhibiting HIF, FIH) enzymes [82, 83]. Hydroxylation of the asparagine residue by FIH controls the alpha subunit's transactivational capacity impeding it from recruiting the transcriptional co-activator p300. The double proline hydroxylation by the PHDs controls the cellular abundance of the alpha subunit by creating a binding site for the von Hippel-Lindau protein (VHL), which further allows proteasomal degradation [81, 84]. In a hypoxic environment, PHD and FIH activities are inhibited since hydroxylation is an oxygen-dependent process, leading to alpha subunit stabilization. The stabilized subunit translocates to the cell nucleus, where it complexes with ARNT and with its co-activator p300, binding to the CGTG nucleotide sequence within regulatory regions known as Hypoxia Response Elements (HRE) present in the promotor sequence of its target genes, consequently activating their transcription [80, 85, 86].

Hypoxia fosters a more aggressive tumor phenotype in several ways, some of which were explored in **Papers I** and **IV**: By (1) promoting epithelial-to-mesenchymal transition (EMT), a pro-migratory cellular program that transforms stationary cells into motile mesenchymal cells via cadherin-molecule switch (loss of E-cadherin expression combined with induction of N-cadherin), mediated by driving genes such as TWIST, SNAIL, ZEB, and TGFB [87, 88]; (2) by activation of immunosuppressive responses via increased expression of PD-L1 T-cell suppressor and CD47 don't-eat-me signal, for example [73, 86]; (3) by the promotion of the angiogenic signaling via HIF-mediated induction of VEGF and endothelin gene [80]; (4) by differentiating myeloid cells into pro-tumorigenic TAMs [81, 89]; and (5) by offering a supportive environment for the dormant cancer stem cells [55, 90]. Disruption of HIF-suppressing elements such as the germline inactivation of the VHL gene is known to cause a cancer syndrome, highlighting its impact on pro-tumorigenic pathway activation [83, 91].

Cancer cells are known to rely on anaerobic glycolysis for energy production even in the presence of oxygen, in a process called the Warburg Effect, thus increasing the rate of glucose uptake and processing it into lactate [92]. This adaptation confers adaptive benefits to tumor cells, especially under hypoxic conditions, in which the utilization of oxidative phosphorylation for ATP production, an $O_2$-dependent (aerobic) process, is not possible. Therefore, in the absence of oxygen, the investment of 1 glucose + 2 $NAD^+$ + 2 ADP + 2 inorganic phosphates yields the formation of 2 Pyruvate molecules + 2 NADH + 2 $H^+$ + a net gain of 2 ATPs + 2 $H_2O$ [81]. Combined with the HIF-1a-mediated induction of lactate dehydrogenase (LDHA), the newly generated pyruvate molecules are catalyzed into lactic acid, generating NAD+ in the process, which is an important cofactor for the maintenance of glycolysis [85]. The lactic acid is transferred to the extracellular space via monocarboxylate transporter 4 (MCT4) located in the cell membrane, being further dissociated into lactate and H+. Extracellular lactate doubles as fuel for normoxic tumor cells, via MCT1-mediated uptake, and is known for stimulating tumor growth and immune evasion. The excessive proton accumulation leads to extracellular acidosis, further driving HIF-mediated malignant progression [92]. Additionally, to efficiently shut down the oxidative phosphorylation, the hypoxic cells also rely on the overexpression of pyruvate dehydrogenase kinase, which inactivates the intracellular pyruvate dehydrogenase via its phosphorylation, avoiding the conversion of pyruvate into Acetyl-CoA, a fuel molecule for the citric acid cycle [81, 93].

Inhibition of the stabilization of HIF 1 and 2 presents an interesting venue for the design of tumor-suppressing drugs. Belzutifan, targeting HIF-2 alpha, was recently approved for the treatment of patients with clear cell renal cell carcinoma driven by VHL loss of function [94, 95]. Moreover, Daprodustat, an inhibitor of the HIF PHD, has also been FDA-approved for the treatment of dialysis patients with anemia due to chronic kidney disease [96, 97]. Other approaches to achieve attenuation of the hypoxic re-

sponse were tried but with limited clinical success [86]. These drugs are not used for other cancers since the hypoxic response also happens in HIF-independent pathways. In most malignant tumors, including GBM, the extent of hypoxia-induced targets goes beyond the expression and stabilization of HIF. Thus, knowing that hypoxic conditioning drives tumor progression and treatment resistance, in **Paper III** we explored the hypoxic modulation over the landscape of targetable surface proteins of GBM cells, for the discovery of potential additional ways to inhibit hypoxia-driven responses.

## Vascularization

As seen above, the signaling for aberrant angiogenesis in GBM is triggered by several aspects of the TME such as hypoxia and signaling from stromal and immune cells. The close association between these factors generates a vicious cycle within GBM TME, where the constant signaling towards angiogenesis gives rise to unstructured and leaky newly formed vessels that fail to deliver oxygen and nutrients, further exacerbating the hypoxic state of the tissue, which in turn, reinforces the promotion of blood vessel formation.

In healthy tissues, angiogenesis starts with a stimulatory factor (*e.g.* VEGF overexpression) that signals for the recruitment of endothelial progenitor cells. The secretion of metalloproteinases by the endothelial and pericyte cells remodels the ECM and the basal membrane, giving space for the sprouting of the new vessel. After the network of neovascular structures is completed, signaling by platelet-derived growth factor receptor and angiopoietin allows for the recruitment of pericytes to provide EC support and the secretion of basal membrane components, thus maturating the new vessels [98, 99]. In rapidly growing tissues such as GBM, vessel maturation is often skipped or poorly made due to altered pericyte activity, lower pericyte coverage, incompletion of the basal membrane, and presence of loose endothelial junctions, resulting in a leaky vasculature with increased permeability than of normal CNS vasculature (*a.k.a* the Blood-Tumor-Barrier), leading to the formation of edema and increased intracranial pressure [99, 100]. In extreme cases, GBM can resort to vessel-mimicry, building acellular vessel-like structures in the ECM as a desperate attempt to enable the flow of blood and other components across the tumor. This process results in a poor matrix-laden structure lacking the characteristic EC lining [98, 99]. The transdifferentiation of tumor cells into endothelial-like cells has been proposed as a contributing mechanism to vessel mimicry and as an important factor impairing the response to antivascular therapies [101, 102]. However, this process remains elusive, and further studies to clarify vessel mimicry and the role of glioma cells in this adaptation are warranted.

In a VEGF-independent process, GBM cells can achieve perfusion during parenchymal invasion via vessel co-opting [66, 98]. In this process, tumor cells of the invasive

tumor front grow and travel along the surface of pre-existing vessels, incorporating them and diverting the blood flow for tumor growth [103]. Moreover, in early hypoxia, a transient increase of the blood flow is achieved via quick HIF1-dependent induction of the inducible nitric oxide synthase (iNOS) enzyme, which produces nitric oxide to induce vasodilation and increase flow capacity [80].

The lack of proper vascularization within GBM hampers the effect of different treatment modalities, contributing to therapeutic resistance and poor patient outcomes. For example, radiation therapy is generally known to be more efficient in the presence of oxygen due to the generation of ROS that form a stable complex with the radiotherapy-induced DNA strand break, inhibiting DNA repair [104]. Additionally, the leaky vasculature consequently protects the cells residing in the hypoxic core, since the poor perfusion impairs the delivery of systemically administered therapies such as chemo and immunotherapies.

Considering the importance of the vascular system to GBM aggressiveness, anti-angiogenic therapies emerged as a promising strategy for tumor management. In 2009, Bevacizumab, a humanized monoclonal antibody therapy targeting the vascular endothelial growth factor-A (VEGF-A) protein was FDA-approved for the treatment of recurrent GBM [105]. Despite initial optimism, numerous trials performed later presented negative results [106, 107], and the therapeutic benefit of bevacizumab for GBM patients remains limited due to several factors. Firstly, the TME harbors a redundancy of angiogenic signaling pathways independent of VEGF-A, allowing tumors to circumvent therapeutic blockade. Moreover, the development of therapeutic resistance mediated by genetic mutations or alternative pro-angiogenic factors further hinders treatment efficacy. These limitations, coupled with the possibility of bevacizumab paradoxically promoting tumor aggressiveness through the induction of hypoxia, signaled the necessity of better strategies for GBM management, setting this and several other anti-angiogenic treatments aside [108]. Lastly, patients treated with higher doses of bevacizumab presented with significant side effects, including potentially life-threatening bleeding and thromboembolic events. Low-dose administration of bevacizumab was, however, associated with increased patient quality of life due to reduced steroid use, allowing relief of corticosteroid refractory edema, and led to improvement in progression-free survival, but not in overall survival, in a retrospective clinical study [109].

## Lipid Metabolism

Lipids are indispensable for tumoral metabolic regulation and crucial building blocks for cell proliferation, migration, and vesicle production in GBM tumors. Unlike healthy brain tissue, studies have demonstrated that GBM tumor cells display a distorted metabolic profile, with a peculiar dependence on the acquisition and utilization of extra-

cellular lipids, as well as reactivation of *de novo* lipogenesis [110]. Considering most cancer cells shut down mitochondrial respiration due to hypoxia and the Warburg effect, triglycerides (*i.e.* three fatty acid (FA) molecules joined to glycerol through esterification) are not mobilized to fatty acid oxidation. Instead, tumor cells package the excess intracellular lipids into perilipin-coated monolayer structures known as lipid droplets (LDs). LDs originate from the endoplasmic reticulum and function as storage units for lipids, mostly triglycerides and cholesteryl esters, that can be directed toward energy production once hypoxia is alleviated [111, 112]. LDs help malignant cells avoid cytotoxicity caused by the circulation and buildup of free cytoplasmic FAs and cholesterol. Furthermore, these reserves serve as traps for polyunsaturated FAs, which are removed from the cell membrane and replaced by saturated and monounsaturated FAs to prevent ferroptotic cell death due to lipid peroxidation [113, 114]. By keeping membrane homeostasis, LDs can also grant ROS protection, contributing to therapeutic resistance to radiotherapy [112]. The LD formation process is facilitated by enzymes such as DGAT1 and SOAT1, which catalyze the final step in triglyceride and cholesterol ester synthesis, respectively. While in the endoplasmic reticulum, these enzymes link diacylglycerol or cholesterol to FAs for efficient packaging of these molecules [111, 113].

In cancerous cells, the abundance of FAs and triglycerides comes from increased lipid uptake and increased lipogenesis. In FA synthesis pathway, the sterol regulatory element -binding protein 1 (SREBP-1) is the main nuclear regulator of the involved genes [111, 114]. In addition to insulin signaling, SREBP-1 is transcriptionally induced by commonly upregulated pathways in cancer cells such as Myc, and growth factors (*e.g.* EGFR) [114, 115]. Moreover, the nuclear accumulation of the mature SREBP-1 protein is known to be induced by the PI3K-mTORC route (which is further induced by HIF-1 signaling) via phosphorylative inactivation of its regulatory proteins, glycogen synthase kinase 3 (GSK3) and Lipin1, driving lipid synthesis during cell growth [114]. The carbon source necessary for lipogenesis is obtained from several exogenously obtained products such as glucose, acetate, and glutamine, resulting in palmitate production that is then properly elongated and decorated for the generation of different lipid species [113]. GBM cells also heavily rely on lipid uptake, which occurs by activation of multiple pathways, such as the endocytosis of low-density lipoproteins (LDLs) mediated by LDL receptors (LDLRs), heparan sulfate proteoglycan, FA scavenging through internalization mediated by FABP4/CD36/FATP, and extracellular vesicle (EV) uptake either through macropinocytosis, receptor-dependent internalization (*e.g.* integrins and syndecans), or membrane fusion [116].

Strategies to target the lipid dependence of cancer cells have been explored on several fronts including inhibition of FA synthesis, blocking of lipid uptake, and interference of lipid storage mechanisms, offering promising avenues for new treatments [113, 118]. Of these, inhibition of the fatty acid synthase (FASN) that catalyzes the key step con-

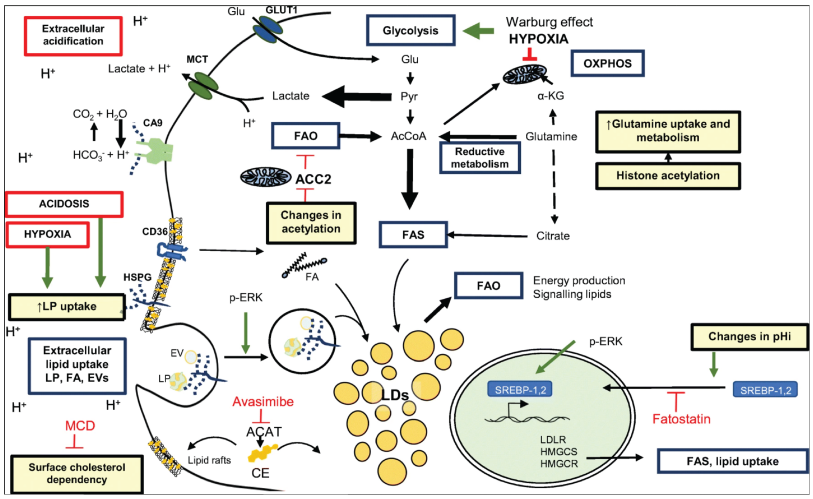


**Figure 5. An overview of the TME.**
An overview of the interplay between hypoxia, metabolism, and lipids. α-KG: alpha-ketoglutarate; ACC2: acetyl-CoA carboxylase; ACAT: Acyl-CoA cholesterol acyltransferase; Ac-CoA: Acetyl CoA; CA9: carbonic anhydrase 9; CE: cholesteryl ester; EV: extracellular vesicle; FA: fatty acid; FAO: fatty acid oxidation; FAS: fatty acid synthase; Glu: glucose; GLUT1: glucose transporter 1; HMGCR: HMGCoA reductase; HMGCS: HMGCoA synthase; LDLR: low-density lipoprotein receptor; LDs: lipid droplets; LP: lipoprotein; MCD: methyl-β-cyclodextrin; MCT: monocarboxylate transporter; OXPHOS: oxidative phosphorylation; pHi: intracellular pH; Pyr: pyruvate; SREBP: sterol regulatory element-binding protein. Reprint with permission from authors (Bång-Rudenstam *et al.*, 2019) [117].

version of malonyl-CoA to palmitate has received significant attention, and several drugs were trialed for the treatment of different cancers [113]. First-generation drugs such as orlistat and cerulenin, showed promising anti-tumoral effects in mouse models but led to excessive systemic side effects during clinical trials with breast cancer patients [113, 119, 120]. The combination of the next-generation inhibitor TVB-2640 with bevacizumab resulted in increased progression-free survival of patients with relapsed high-grade astrocytoma in a phase II clinical trial [121]. Moreover, TVB-3166, showed promising anti-tumoral effects in preclinical models of colorectal and breast cancers [119, 122, 123]. Besides FASN, inhibition of SOAT1 and DGAT1, which are the proteins contributing to LD formation, has been shown to induce cytotoxicity via excess lipids or cholesterol within the endoplasmic reticulum, causing lipotoxicity, suppressing GBM cell growth [124, 125]. Moreover, DGAT1 inhibition can further induce oxidative stress and, consequently, ferroptosis. Other targetable proteins for lipid disruption in cancer cells are SDC1 (to impair HSPG-dependent lipoproteins and EV uptake), LPCAT2 (for further LD-formation disruption), ACLY and ACSS2 (to inhibit the conversion of citrate or acetate into Acetyl-CoA, respectively), among other proteins, encouraging further efforts towards cancer cell eradication [113, 118].

# Challenges

GBM is a particularly challenging tumor to treat, with several factors contributing to the difficulty in achieving successful treatment outcomes. The highly infiltrative nature of GBM makes complete surgical resection nearly impossible, and the remaining malignant cells surely regrow the tumoral mass, which is usually unresponsive to additional rounds of treatment. Moreover, the BBB poses another significant hurdle for GBM treatment due to their rigorous regulation over the paracellular transport of blood-borne substances [67]. While this function is vital for maintaining a stable neuronal environment, it can block therapeutic drug delivery, since several classes of chemotherapeutic agents are unable to penetrate the BBB effectively, limiting their efficacy in targeting tumor cells.

Further complicating the therapeutic landscape is the hypoxic and acidic microenvironment within GBM tumors that contributes to the selection of therapy-resistant tumor cells via selective pressure. Consequently, tumors exhibit significant variability on a genetic and molecular level, rendering therapeutic strategies ineffective against most subpopulations of tumor cells. Moreover, GBM is known to foster an immunosuppressive microenvironment via the secretion of several factors that actively suppress the immune system's ability to mount an anti-tumor response [126]. For instance, the tumor induces the release of proteins such as transforming growth factor-beta (TGFB) which inhibits the proliferation and cytotoxic activity of T-cells, creating an environment where cancer cells can evade immune detection and elimination. [38]

Finally, the presence of cancer stem cells within GBM tumors and the interplay with the TME presents additional hurdles. Cancer stem cells are thought to be resistant to conventional therapies and may contribute to tumor recurrence after treatment. Meanwhile, the communication between tumor cells and their microenvironment is known to promote angiogenesis, cell migration, EMT, and other pro-tumoral processes that further diminish the patient´s chances of long survival despite the therapeutical course of action implemented [73]. Thus, overcoming these challenges requires continued research into new drug delivery systems across the BBB, targeted immunotherapies, and the design of clever strategies to address tumor heterogeneity, invasiveness, and the presence of cancer stem cells.

# Treatment Modalities

The choice of the ideal therapeutic line of action for each GBM patient is impacted by a range of factors such as patient age, overall patient health status, location of the tumor within the brain, presence or absence of specific tumoral biomarkers, availability of treatment modalities within the care facility, etc. The decision-making process is multidisciplinary and should be tailored to each patient, taking the professional's input as well as the patient's goals and preferences into consideration [127]. Unfortunately, there is still no curative therapy for GBM, and despite exhaustive research efforts, patient survival is yet limited, usually not surpassing 15 months.

This section showcases some of the treatments currently in use, as well as some promising modalities under development. It is important to note that, rather than fixed, the current methods here presented are also undergoing constant improvements (*e.g.* the combination of intratumoral delivery with radio and immunotherapy for improved targeted delivery of radiolabeled antibodies [128]) to increase the treatment's efficacy and/or decrease adverse effects for patients.

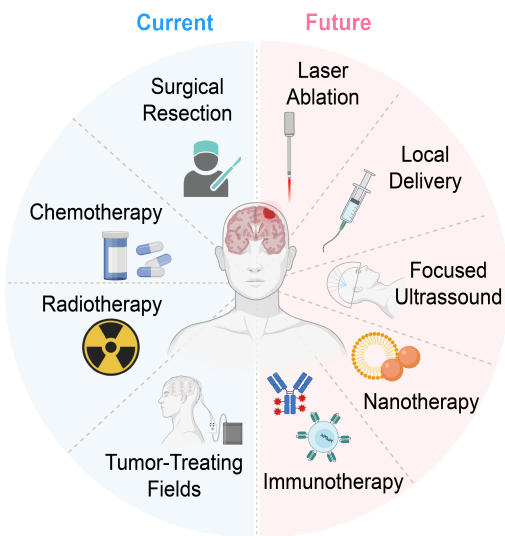


**Figure 6. Current and future treatment modalities for GBM.**
Made with BioRender.

## Current Treatments

### Surgery

Maximal resection of the tumor bulk is the first step in the treatment plan against GBM, and it aims to reduce/stop any further displacement, compression, and injury of the healthy brain tissue caused by the growth of the tumor (*i.e.* the mass effect) [129], as well as to improve the efficacy of administered adjuvant therapies [79]. Complete removal of the GBM tumor burden is difficult to achieve due to the high degree of infiltrating tumor cells, which spread out into different brain regions, leading to unclear tumor borders and the permanence of undetectable cancerous cells that inevitably lead to tumor recurrence [2, 130]. According to several studies evaluating the impact of the extension of surgery on patient prognosis [131–133], even though radical surgical resection does not guarantee progression-free survival, maximal removal of tumoral tissue (>80% of the tumor volume) improved survival in patients [134]. However, these patients were more prone to the development of neurological morbidity within the first 30 days post-surgery, highlighting the need for a balanced assessment on a per-case basis.

In addition to surgical extension, technical improvements such as fluorescence guiding also currently assist surgeons in removing GBM. This technique benefits from the preferential accumulation of 5-aminolevulinic acid (5-ALA, *i.e.* a hemoglobin precursor) within metabolically hyperactive cells. The 5-ALA dye is administered to patients as an oral solution 2-3 hours before surgery [135], and accumulates into malignant cells, causing them to fluoresce under a fluorescence microscope and enabling the visualization of cancer cells during surgery. Moreover, other approaches performed during surgery such as intraoperative ultrasound and intraoperative radiotherapy may assist the chase against tumoral cells by providing real-time tumor images and by reducing the interval between removal of the tumor and initiation of adjuvant therapies, respectively.

### The Stupp protocol

Oncological treatment of GBM shows national and even regional variations. According to current guidelines in Sweden [136], in addition to the maximal surgical tumor resection, the standard treatment for GBM patients aged around 70 years or younger (with good overall performance) includes a therapeutic combination known as the Stupp protocol, in which the radiation therapy regime is simultaneously administered with chemotherapy [137, 138]. In this protocol, named after the Swiss oncologist Roger Stupp, once the surgery is performed, the patient undergoes daily fractionated focal irradiation sessions (2 Gy doses, 5 days per week for 6 weeks) and concomitantly re-

ceives oral doses of Temozolomide (TMZ), a DNA alkylating agent, for 7 days per week, during these 6 weeks (75 mg/m$^2$/day). In the next step, after the rounds of radiotherapy are concluded, the patient receives 6 cycles of adjuvant TMZ given at higher doses of 150 to 200 mg/m$^2$/day during 5 days/cycle/ for 28 days.

Despite potential side effects (*e.g.* thrombocytopenia, neutropenia, fatigue, liver toxicity, infections, etc), the Stupp protocol has significantly improved survival rates for GBM patients. Before its introduction, radiotherapy was often the only treatment option, and patients presented a median survival of 12.1 months. With the addition of TMZ during the radiotherapy round, median patient survival has been extended to 14.6 months [137], remaining the most effective treatment regimen for GBM identified thus far and standing as a landmark discovery in the history of GBM treatment.

Due to the impact of MGMT promoter methylation status in predicting the response outcome to TMZ, the administration of hypofractionated (34 Gy in 10 fractions) radiotherapy rounds alone can be considered for patients presenting tumors with unmethylated MGMT promoter [138]. In fact, patients with MGMT unmethylated tumors should all be considered for clinical trials with alternative treatments. Furthermore, for patients older than 70 years or with poor performance, tolerance to radiotherapy can be considerably lower, thus, the administration of TMZ alone can also be a reasonable option in cases of MGMT-promoter methylated tumors. Finally, elderly patients with MGMT methylated tumors but in good performance status are recommended combined TMZ and radiotherapy with an intermediate dose fractionation (40.05 Gy/15 fractions) [139]. Together, this underscores the importance of a modulated protocol, tailored to individual patient and tumor characteristics, to optimize treatment efficacy and minimize side effects [138].

### Tumor Treating Fields

Tumor-treating fields (TTF) emerged as an antimitotic therapeutic modality introduced in some countries, including Sweden, at the maintenance stage of GBM treatment [140]. By making use of low-intensity alternating electric fields, TTF disrupts several cellular processes such as cell division, DNA repair, and neovascularization, potentially inhibiting tumor progression and extending patient survival. According to a phase II clinical trial, TTF displayed improved patient survival in 5 months, showing potential as a complementary method to the traditional treatments [141, 142].

### Chemo-radiotherapy

Beyond the established Stupp protocol, alternative chemotherapies have been utilized for GBM treatment. In newly diagnosed GBM patients with lower tolerance to radiotherapy, the combination of TMZ with lomustine, an alkylating chloroethylating nitrosoureas, displayed promising survival benefits (approx. 17 months increase in median overall survival) [143]. Beyond DNA alkylation, lomustine affects post-transcriptional processes by causing carbamoylation of amino acids and further induction of DNA damage by the introduction of inter-strand crosslinks [144]. For the treatment of patients with recurrent GBM, lomustine and other nitrosoureas, such as carmustine (intravenous or as wafers placed in the tumor cavity after resection), may be utilized, especially against MGMT methylated tumors [8].

Regarding other approaches for the improvement of radiotherapy, the use of MRI-Linac has shown some promise. It combines MRI scan-based imaging with linear accelerators for tumor irradiation, enabling real-time tracking of the tumor borders and volume for precise directing of the radiotherapy beam, and decreasing off-target radiation delivery and damage to healthy surrounding tissue [145].

## Future Treatment Possibilities

### Laser ablation

Laser ablation, also known as laser interstitial thermal therapy (LITT), is an alternative surgical tool particularly useful in cases where open craniotomy cannot be performed. The procedure utilizes a focused laser beam to directly heat and safely ablate tumor tissue while minimizing damage to surrounding healthy brain structures. In GBM, around 15-25% of patients do not receive surgical resection, only undergoing tissue biopsy, due to surgical risk (*e.g.* when tumors are in close proximity to critical brain regions) or personal preference. In these cases of inoperable tumors, MRI-guided laser ablation could be utilized for the destruction of the malignant tissue in a less invasive way, providing faster post-procedure recovery and potentially offering an overall survival similar to that of patients submitted to traditional open surgery [146]. The results of ongoing and planned clinical trials will shed more light on the usefulness of LITT.

### Local Delivery Strategies

As mentioned in the section **Challenges**, the BBB significantly limits the efficient treatment of GBM by restricting the delivery of therapeutic agents to the tumor site. To

overcome this hurdle, local delivery strategies are explored as a promising avenue in GBM treatment for allowing the direct deposit of concentrated therapeutic drugs within the tumor cavity or surrounding brain tissue while limiting the systemic adverse effects [147]. One of the methods utilized to accomplish this is the stereotactic injection, which utilizes a three-dimensional coordinate system generated by imaging data, such as MRI or CT scans, to guide the precise placement of a catheter into a targeted region within the brain, enabling the delivery of substances such as therapeutic drugs, nanoparticles, or ablative agents directly to the site of interest with minimal damage to surrounding tissues [147]. In contrast to stereotactic injection, convection-enhanced delivery utilizes pump-attached catheters implanted within the designated area to create a slow, pressurized infusion of therapeutic agents, that actively distribute the drug throughout the surrounding tissue and achieves higher and more uniform concentrations compared to diffusion alone [148].

Implanted ventricular reservoirs, such as the Ommaya reservoirs, function as refillable depots for sustained and controlled intrathecal drug delivery over extended periods. Even though the low bulk flow in the cerebrospinal fluid allows therapeutics to diffuse into the parenchyma, the delivery to the intraparenchymal GBM tumor site is not effective [147, 149]. Alternatively, to overcome this issue, the catheter can be placed directly into the tumoral cavity. Lastly, intra-arterial delivery systems represent a specialized method for drug administration that bypasses systemic circulation, being usually combined with temporary BBB disruption (*e.g.* through the use of focused ultrasound. See below.) to improve the passage of the delivered drug [147]. In this approach, a catheter is image-guided through the arterial network until it reaches the feeding artery supplying the target tissue. However, this technique requires precise catheter placement and carries inherent risks such as arterial punctures and potential embolization of blood vessels. Alternatively, the catheter can also be placed into the tumoral cavity or ventricles [147]. In conclusion, further development of different local delivery systems for GBM patients is a necessary way forward to allow for therapeutic tailoring without concerns over the drug's BBB crossing potential and systemic off-target toxicity, consequently improving the patient's life quality and prognosis.

### Focused Ultrasound

Focused ultrasound (FUS) in conjunction with intravenously injected microbubbles allows guided and reversible disruption of the BBB. Preclinical studies demonstrated the safety and efficacy of FUS-mediated BBB opening [150]. FUS enhanced the accumulation of chemotherapeutics, nanoparticles, and therapeutic cells in the TME, reduced tumor progression and improved survival in mice [151]. Recent clinical trials showed the feasibility and safety of repeated FUS-mediated BBB opening as well as enhanced

intratumoral uptake of cytostatics in GBM [152]. The technique is further being improved to allow for larger BBB disruption areas [153], and clinical trials to assess its impact on therapy efficiency are warranted. FUS thus emerges as a promising, non-invasive, spatially precise technique for transiently breaching the BBB.

### Nanotherapy

Tumor nanotherapy represents a nano-scaled branch of the macro local delivery systems discussed above. It comprises the utilization of engineered nanoparticles (NPs) (ranging between 1-400 nm in size) as carriers to deliver drugs specifically to the tumor cells within functioning brain regions without affecting their neighboring healthy cells [154–156]. Nanotherapy has the potential to overcome several limitations posed by conventional chemotherapy delivery. The encapsulation of therapeutic drugs and the surface characteristics of NPs allows them to exert precise targeting of tumor cells, modulate drug biodistribution, extend drug half-life, improve target site accumulation, enhance therapeutic efficacy through secondary events (*e.g.* ROS induction, activation of an inflammatory response, etc), and reduce off-target adverse effects [154, 157]. The particles utilized by nanosystems can be built similarly to biological vesicles (*e.g.* exosomes and micellar emulsions) or from compounds such as polymers, metals, silica, carbon dots, nano-gel, liposomes, among many other available materials [41, 158]. Antitumoral agents such as chemotherapeutics, radiosensitizing drugs, and immune cells can be loaded into the NP, and its surface can be decorated with target-binding domains (for specific tumor cell targeting), fluorescent labels (for particle tracking), and moieties to facilitate passive transport or transcytosis-mediated BBB crossing [41, 158, 159].

Despite extensive pre-clinical exploration of NP-based delivery systems for GBM treatment, only NanoTherm, which utilizes iron oxide NPs, has received the European Medicines Agency approval for magnetic field-induced GBM thermal ablation. Several phase I and II clinical trials have been conducted but most with limited achievements. For example, a pegylated nanoliposomal irinotecan formulation (Onyvide, MM-398) was investigated in a phase I dose-finding trial (NCT03119064) in combination with TMZ treatment administered to twelve patients diagnosed with recurrent GBM, where the concurrent administration of Onyvide with TMZ (both at dosage of 50 mg/m$^2$) was seen to be well-tolerated, although without significant efficacy [154, 160]. Currently, only 2 clinical trials are recruiting patients for the testing of NP-based therapies in GBM (NCT04881032 and NCT06271421) and the benefits of nanotherapeutics in GBM treatment are yet to be confirmed.

### Immunotherapy

Immunotherapy is a highly effective treatment strategy for a wide range of tumors such as breast [161], head and neck [162], melanoma [163], lung cancer [164], and many other malignancies. This approach aims at enhancing the patient's immune system for better anti-tumoral activity or at employing immune effector molecules, such as antibodies, to target and eliminate cancer cells.

In recent years, the interest in immunotherapeutics for GBM treatment has increased sharply. Characterized by low immunogenicity and inherent immunosuppressive properties, GBM is an immunologically cold tumor that effectively evades the host's immune response. Consequently, several immunotherapeutic strategies are being developed or explored towards different tumoral aspects to "warm-up" the immune response of GBM and achieve tumor cell eradication.

**Immune checkpoint inhibitors:** Designed to decrease cancer-associated immuno - tolerance, immune checkpoint inhibitors (ICIs) (Figure 7) are another monoclonal antibody-based therapy clinically tested in GBMs. Immune checkpoints are molecular receptors that suppress immune cell activation to prevent them from going into overdrive and GBM tumors express complementary proteins that interact with these receptors, further encouraging suppression and immune cell evasion [165]. Thus, ICI-mediated blocking of CTLA-4 and PD-1 receptors (*i.e.* immune checkpoints expressed at the surface of T cells) with monoclonal antibodies such as ipilimumab and nivolumab, respectively, could trigger robust T-cell attack and induce tumor regression, giving hopes of a breakthrough therapy for GBM patients. Despite this being true for some cancers, during GBM clinical trials (*e.g.* NCT02017717, NCT02311920, NCT02829931), no breakthrough survival benefits were observed with the use of this type of immunotherapy, possibly due to low tumor T-cell availability [126, 166, 167].

**Antibody-Drug Conjugates:** Antibody-drug conjugates (ADCs) (Figure 7) have recently been established as a new drug category for oncological treatments within the field of immunotherapy. The pioneering concept of "magic bullet" ADCs emerged in the early 1900s. Technological advancements in chemistry and the development of monoclonal antibodies enabled the construction of the first conjugates in the 1970s. Despite early challenges in the 20th century concerning problematic toxicities, the 21st century witnessed the first FDA-approved ADC, gemtuzumab ozogamicin (2000). This success paved the way for subsequent ADC approvals such as brentuximab vedotin (2011) and ado-trastuzumab emtansine (2013) [168]. Currently, there are 15 FDA-approved drugs for the treatment of *e.g.* breast, ovarian, and bladder cancer, and hundreds of clinical trials are ongoing [169]. ADC is a class of pharmacological compounds comprised of a monoclonal antibody designed to target tumor-associated antigens, covalently conjugated via a stable linker to a cytotoxic drug. This targeted

approach allows ADCs to selectively bind and deliver the cytotoxic agent directly to tumor cells, promoting their eradication, consequently offering a more favorable therapeutic index compared to conventional untargeted approaches. This translates to potentially fewer systemic side effects and improved patient tolerability [170, 171]. While the choice of the targeted protein and the specificity of the antibody warrants the selective targeting of cancer cells, the attached cytotoxic drug (*a.k.a.* the payload or warhead) and linker structure are crucial for guaranteeing effective cell death either upon ADC endocytosis by the cancer cell or through bystander effect [172]. The ADC payloads, which are usually tubulin inhibitors (*e.g.* Monomethyl auristatins and maytansinoid derivatives) or DNA-damaging drugs (*e.g.* Duocarmycin and Calicheamicin), exert a potent effect when internalized by cells while remaining stable when in extracellular space [170]. Moreover, depending on its polarity, free payloads can display higher or lower membrane permeability, which is used to modulate the bystander effect potential of each drug [170, 171]. Meanwhile, the linker, categorized as cleavable (*e.g.* disulfide linkers) or non-cleavable (*e.g.* thioether linkers), is of equal importance to the ADC's success. It should be stable to prevent the premature release of the cytotoxic drug in the plasma, therefore preventing systemic toxicity, but also degradable by specific events such as by enzymatic degradation (*e.g.* via cathepsins), by the low intracellular pH, or by complete lysosomal-driven protein degradation [172, 173]. The epidermal growth factor receptor (EGFR) amplification frequently observed in GBM (about 50% of newly diagnosed patients present EGFR alterations) is in the spotlight for ADC trials against GBM. However, the attempts have not yet resulted in clinical success, and recent studies utilizing ADCs against both the wildtype and the EGFRvIII mutant protein led to no increase in overall survival when compared to patients that received standard treatment and displayed concerning adverse effects [174]. In **Papers II** and **III**, we develop a new strategy for the identification of potential ADC targets and set up protocols for assessing ADC treatment effects *in vitro*.

**Additional functionalization of targeting antibodies:** Beyond the conventional targeting achieved by monoclonal antibodies in ADCs, further functionalization strategies are being explored to enhance therapeutic efficacy. Bispecific antibodies, *i.e.* molecules that possess two antigen-binding domains to bind to two distinct targets simultaneously, represent a promising approach. These antibodies can bridge tumor cells with immune effector cells for a more potent anti-tumor response. For example, Bispecific T cell Engagers can activate T lymphocytes to promote lysis of target-antigen expressing tumor cells [41, 126]. Additionally, the engineering of antibodies to recruit innate immune effector cells via activation of Fc$\gamma$ receptors is another strategy gaining momentum. Antibody-Dependent Cellular Cytotoxicity (ADCC) (Figure 7) harnesses the cytotoxic potential of NK cells, while Antibody-Dependent Cellular Phagocytosis (ADCP) (Figure 7) leverages the phagocytic activity of macrophages [164]. Moreover, Radiolabeled monoclonal antibodies (Figure 7) can further enable targeted imaging and help

improve the potential of targeted radiotherapy delivery [41]. These functionalized antibodies hold promise for broadening the immune attack on cancer cells and potentially overcoming tumor immune escape mechanisms, offering other therapeutic avenues for the treatment of GBM patients.

**Cancer Vaccines:** Differently from the monoclonal antibody-based techniques, cancer vaccines rely on the administration of tumor-specific antigens (peptides, DNA, or mRNA), usually conjugated to an immunogenic protein for immunological response stimulation. Rindopepimut, a peptide vaccine comprised of EGFRvIII-specific peptides conjugated to a xenogeneic metalloprotein, was trialed on a GBM cohort in phase-II trials, displaying promising results (a survival increase of 11 months in the treated arm when compared to control) [43]. However, phase-III trials showed no difference between the study arms [126, 175]. Cancer vaccines based on mRNA-transfected dendritic cells such as the DCVax-L, where autologous dendritic cells are expanded and primed with tumor lysates, proved to be a safe line of action for GBM patients, displaying increased overall survival when administered in combination with standard of care in a phase III non-randomized single-arm trial with an external control group treated only with standard of care [126, 176]. This outcome encourages further research, especially involving tumor neoepitopes, and shows that the landscape of effective GBM treatments can still increase in the future.

**Oncolytic Virus Therapy:** In another strategy, known as oncolytic viruses, weakly pathogenic genetically modified viruses selectively infect, replicate, and destroy tumor tissue while triggering the immune system [73, 126]. Clinical trials have shown exciting tumor-suppressing effects such as immunogenic death of malignant cells, release of tumor-associated antigens (TAAs) and molecular patterns (DAMPs and PAMPs), activation of the innate immune response, as well as stimulation of T cell infiltration into the tumor region [126, 177–179]. Notably, a phase-II single-arm clinical trial investigating G47-delta, a triple-mutated oncolytic herpes simplex virus 1 (HSV-1), for recurrent or residual GBM demonstrated promising efficacy and safety [180]. This significant improvement made G47-delta the first oncolytic treatment to be approved by the Japanese administration, encouraging researchers in the field to further explore oncolytic viruses for GBM treatment.

**Chimeric Antigen Receptor T-cell:** Lastly, Chimeric Antigen Receptor (CAR) T-cell (Figure 7) therapy involves the reintroduction of lentivirus-engineered patient T cells for recognition of tumoral antigens and targeted attack of malignant cells. A constructed chimeric receptor (CAR) protein is comprised of an extracellular domain (scFv) that selectively binds to a tumor antigen, fused to a transmembrane region, followed by a CD3-derived intracellular signaling domain responsible for cytotoxic response initiation, and co-stimulatory regions that contribute to T-cell persistence. Upon reinjection, CAR T-cells rapidly multiply and its binding to the specific TAA initiates

CAR molecule clustering, and activation of the cytotoxic cascade [13, 73, 126, 165]. This is a promising therapy already in use for the treatment of hematological tumors but is still under development for application to solid tumors. The main targets currently explored for CAR T-cell development in GBM mouse models are the human epidermal growth factor receptor 2 (HER2), the oncogenic EGFRvIII variant, the wildtype EGFR, and the alpha receptor 2 of IL-13 (IL13RA2) [13, 73, 126]. In 2021, one patient was reported to live 36 months after GBM recurrence during a trial testing an anti-EGFRvIII variant CAR T-cell, after having received a single treatment infusion [181]. In a recent study, further targeting of EGFR (both variant and wildtype) by Choi *et al.* [182] displayed rapid tumor regression, albeit transient, and Bagley *et al.* [183] reported early results of a phase I trial with 6 patients receiving bivalent CAR T cells targeting EGFR and IL13RA2, and signs of initial tumor reduction were observed. Also, a completed phase I trial with 65 patients with recurrent GBM treated with IL13RA2-targeted CAR-T cells indicated a possible benefit for patients receiving the maximal feasible dose [184] but more advanced trials are needed to assess proper tumor reduction, since the scope of phase I trials is the assessment of dose-related safety. The main challenge regarding CAR T-cell adaptation to fight GBM lies in tumor heterogeneity, which limits the approach's efficiency to a subset of cells expressing the targeted surface antigen. In this sense, further efforts are being put into the refinement of CAR T cells for the induction of bystander effects via, for example, antigen spreading that occurs once an active lymphocyte attracts other pro-inflammatory innate immune cells, inducing T helper maturation and dendritic cell polarization to exert antitumoral activity towards antigens not originally targeted. CAR NAP T-cells could potentially achieve this goal through the combined expression of the neutrophil-activating protein (NAP) from Helicobacter pylori, which was observed to trigger pluripotent pro-inflammatory bystander T-cell responses when tested against solid cancers [185], holding potential to significantly improve the outlook for GBM patients. While highly effective, CAR T cell-based therapies can be associated with severe adverse effects such as neurotoxicity, requiring careful patient monitoring and management.

# Surface proteins and tumor antigens

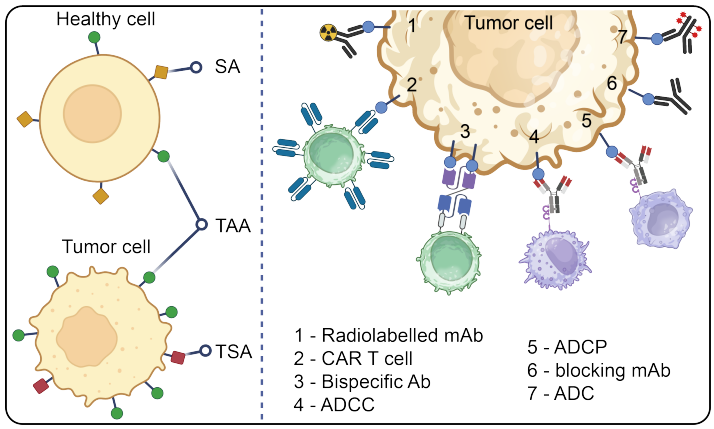


**Figure 7. Surface antigens and targeting strategies.**
**A)** Antigen types: Self Antigens (SA): proteins found in healthy cells; Tumor-Associated Antigens (TAA): proteins found both in healthy and tumor tissues, usually overexpressed in the tumor; Tumor-Specific Antigens (TSA): proteins found exclusively in tumoral cells. **B)** Applications of tumoral antigens for therapeutical targeting. Made with BioRender.

As seen in the section **Treatment Modalities**, many of the established and experimental therapeutical fronts against GBM are moving towards the inclusion of specific targeting strategies as a promising way to improve their therapeutical efficacy. To achieve this precise steering of antitumoral agents to tumor cells, most techniques, *e.g.* CAR-T cell and ADC, rely on the identification of exclusive or abundantly expressed surface proteins.

Understanding the landscape of surface proteins expressed by diverging cellular entities and how these are modulated by different events (*e.g.* hypoxia, chemotherapy, etc) allows for the leveraging of their unique molecular profile towards better targeting of cancer cells (or TME features) while minimizing the toxicity to healthy tissues. However, identifying biologically relevant and targetable tumor surface antigens for therapeutical design is not a trivial task. The heterogeneity amongst GBM cells, the presence of signaling pathway redundancy, and adaptive responses to microenvironmental stresses make the discovery of meaningful proteins a challenging process. For example, the high degree of intratumoral heterogeneity seen in GBM tumors hampers the targeting of a broad range of cellular subclones by the same approach since most

subclones present with distinct molecular signatures than that covered by the targeting treatment. Moreover, the presence of redundant routes impairs the effective blocking of pro-tumorigenic mechanisms, since these restore the inhibited signaling pathway through compensatory channels.

Surface proteins expressed uniquely by tumoral cells, *i.e.* tumor-specific antigens (TSAs) or neoantigens, would be the ultimate target for precision medicine strategies. However, neoantigens are relatively limited events in GBM due to their low mutational burden, and the expression of the TSA can be limited to a few cell subpopulations, which would hinder the efficacy of the treatment caused by the lack of homogeneous tumor expression. Consequently, the search for eligible proteins for directed approaches commonly focuses on the discovery of appropriate tumor-associated antigens (TAAs), which are proteins overexpressed by tumor cells but also found at lower levels in normal cells. Thus, in this section, we will briefly dive into the efforts and some of the available tools utilized in the search for these antigens at different molecular levels.

## Genomics

The adaptive potential of cancer cells comes partly from the plasticity of their genome. The tumoral DNA accumulates aberrations due to deliberate impairment of the DNA damage repair system, in attempts to grow, invade, and survive [186]. While some alterations result in death induced by genomic instability, others result in the emergence of novel protein versions (*e.g.* TSAs) or overexpression/silencing of genes.

The most common types of genome alterations seen in tumors are gene amplification (*i.e.* duplication of the whole gene sequence within the chromosome), gene truncation (which can be caused by large-scale deletion of a gene segment or due to mutations), and somatic mutations (*i.e.* post-fertilization changes in the DNA sequence), leading to the production of altered protein forms [187]. Notably, GBM often exhibits amplification of tyrosine kinases such as EFGR and MET genes, as well as inactivation of tumor suppressor genes by mutation (*e.g.* TP53), or truncation (*e.g.* PTEN), causing the suppression of cell cycle regulation and aberrant activation of proliferative routes [188, 189]. Furthermore, tumors can exhibit neoantigens arising from these alterations, but also as a consequence of gene fusions, insertions, or deletions [187].

The profiling of tumoral DNA alterations can be performed through several methods. For example, to profile deletions, duplications or even complete losses of specific DNA segments, copy number variation analyses can be done after sequencing the tumor's genome, revealing potential driver mutations involved in tumor development. Moreover, variant calling analysis of DNA sequencing data from both tumoral and healthy cells allows for the detection of tumor-specific mutations. In a 2019 phase I/Ib study,

Keskin *et al.* utilized this approach for the identification of nucleotide variants leading to single amino acid missense mutations and small insertions/deletions, followed by HLA-binding analysis for the generation of a multi-peptide neoantigen vaccine. Eight newly diagnosed GBM patients received the vaccine dose weeks after surgical resection and conventional radiotherapy [190]. Despite good tolerability, only two patients developed immune responses towards neoantigen peptides and overall survival reached 16.8 months, illustrating the limited benefit of this approach.

As described in the subsection **Cancer Vaccines** from **Treatment Modalities**, the tumor-specific EGFR variant was also utilized for the construction of the Rindopepmut peptide vaccine, which, despite targeting a protein form that is abundantly expressed by GBM cells, displayed no survival benefit in phase III trials [175]. Albeit interesting, DNA-based approaches are limited in the identification of tumor (specific or associated) antigens or surface targets. Specifically, there is uncertainty regarding the extent to which amplifications or mutations identified at the DNA level translate into actual protein expression. Furthermore, upon identification, the DNA information does not reveal whether the observed effects on protein expression are of sufficient magnitude to warrant the development of peptide vaccines or immunotherapies such as ADC or CAR-T cell strategies. Moreover, cancer cells and immunosuppressed macrophages are known to tune down peptide presentation, limiting immune response generation.

## Transcriptomics

Quantification of gene expression through measurement of available transcripts provides a comprehensive snapshot of gene regulation between samples of different conditions. In cancer research, some of the most significant contributions of transcriptomics are the identification of tumoral biomarkers (*i.e.* genes or proteins that can be used to diagnose, monitor, or predict the outcome of cancer) and the development of targeted therapies (*i.e.* the identification of tumor-associated genes for the design of therapeutic interventions that specifically target the gene or its protein product).

Similarly to genomics, while RNA-based approaches are beneficial for the exploration of tumorigenesis regulation, the clinical implementation of strategies toward tumor-associated surface targets discovered through RNA approaches can be limited since the technique does not confirm the presence of the end protein within the tissue. While mRNAs are a step closer to actual protein production than DNA, their splicing, exportation, translation, and degradation are heavily modulated by molecular alterations and the presence of sponge non-coding RNAs [191, 192].

In **Paper III**, the comparison between the abundance of expressed tumor-associated surface genes measured by RNA-seq of GBM cells and tumor-associated surface pro-

teins measured by label-free proteomics showed a poor correlation between gene to protein levels of surface TAAs, indicating that mRNA is an inaccurate surrogate for protein expression of targetable surface entities. Yet, studies have shown that the heterogeneity of RNA events (*e.g.* alternative splicing and transcript fusions) taking place in tumors can give rise to several novel TSAs, rendering RNA a strong contributor associated with the diversification of the cancer neoantigen proteome repertoire [193, 194]. The generation of neoantigens originating from skipped exons (*a.k.a.* neo-junctions) is believed to be shared among patients and to happen with higher frequency in tumor cells than those from single nucleotide mutations [187], giving hope for the existence of better neoantigen sources to be tapped for the development of targeted therapies. The exploration of RNA neoepitopes is still in its early phase and has been studied, so far, commonly in combination with DNA-generated neoepitopes.

## Proteomics

Proteins are involved in nearly all cellular processes taking place in a living organism. Proteomics (*i.e.* the study of the structure or mapping of the genome-coded proteins) provides valuable insights into the state of a given tissue and its use has rapidly grown amongst the multi-omics sectors. The number of proteomic-related publications deposited in PubMed Central (PMC, National Institute of Health, accessed in 2024) across the last decade increased by approximately 70% in 2023 compared to 2013. Since abundant expression of a gene does not always correlate to abundant production of its corresponding product, proteomics enables a more precise look into the mechanisms of complex conditions, helping dissect the relationship between genotype and phenotype.

Proteomics is represented by a myriad of low and high-throughput techniques ranging from 2D gel-assisted methods to advanced methods such as nuclear magnetic resonance and mass spectrometries [195]. For the study of surface proteins, profiling of whole cell lysates is not optimal due to the underrepresentation of surface proteins when in a mixture with cytoplasmic content. Thus, enrichment methods are necessary to isolate surface proteins before proteomic analysis. Subcellular fractionation, a method utilized at the rise of proteomics, separates cellular components, allowing for the targeted analysis of plasma membrane proteins. Additionally, glycoprotein-based enrichment strategies were also utilized, and capitalized on the frequent glycosylation of surface proteins, enabling their selective isolation and identification [196]. Nowadays, other methods for surface protein enrichment have been developed such as chemical labeling isolation and antibody libraries [197].

To date, protein profiling is a strong ally to the discovery of tumor-associated surface targets, being implemented in **Papers I-III**, for example, for the identification of over-

expressed proteins in cancer patient cells and cell lines subjected to TME-mimicking conditions. Proximity Extension Array (PEA, see **Methods**), a method applied in **Paper I**, was implemented by Álvez *et al.* [198] to profile protein signatures in the plasma of patients with different types of tumors. Moreover, Hosseini *et al.*[199] describe the utilization of a localized surface plasmon resonance sensor chip for the proteomic detection of infinitesimal amounts of exosomal surface biomarkers, as a glioma biosensor. Interestingly, neoantigens can also rise from aberrant post-translational modifications (PTMs) due to the generation of novel N-glycosylation sites resulting from mutations [187], which can be profiled through glycoproteome approaches.

While advancements in mass spectrometry instrumentation and data analysis pipelines are continuously improving sensitivity and accuracy, proteomics still presents several challenges. One such limitation is the inherent dynamic range of the proteome, where highly abundant proteins can mask the detection of lower-abundance antigens. Additionally, the identification of specific PTMs on neoantigens can be technically demanding, requiring specialized enrichment strategies and robust bioinformatic tools for confident peptide/PTM assignment. Furthermore, unlike mass spectrometry-based proteomics, PEA relies on the specificities of antibodies, potentially leading to underestimation of the surface proteome due to limitations in antibody availability or recognition of novel or post-translationally modified antigens. Also, the choice of the appropriate technique for the evaluation of surface proteins in a 3D context needs to be carefully done as utilization of an antibody-based approach would probably result in poor characterization due to lack of specificity or cross-reactions. Thus, for profiling 3D structures, as well as capturing endocytosed proteins (whose profiling is necessary for the identification of targets interesting for ADC strategies), well-designed biotinylation-based approaches are more suitable, as developed in **Papers II** and **III**.

## Multi-omics target analysis

Due to the remarkable heterogeneity of GBM, a single omics approach often proves insufficient for uncovering the mechanisms underlying tumorigenesis and identifying therapeutically relevant targets. Thus, more recently, multi-omics efforts emerged from the necessity of layering the different levels of information for visualization of the bigger picture. The implementation of multi-omics strategies, encompassing genomics, transcriptomics, proteomics, and even metabolomics, lipidomics, and methylomics allows the achievement of a more comprehensive understanding of tumoral regulation and might also be the necessary step toward the reliable identification of therapeutically relevant target proteins.

Hinojosa *et al.*, for example, utilized the combination of DNA, RNA, and proteomics to uncover medulloblastoma-restricted neoantigens for the development of personal-

ized T-cell immunotherapy, and primed T-cells displayed *in vitro* cytotoxicity against tumoral cells [193]. In an exciting GBM study, Hilf *et al.* also made use of DNA/RNA/proteomics for the design of two personalized vaccines tested in a phase I trial [200]. The first vaccine, named APVAC1 contained a library of non-mutated individual antigens (TAAs), while the second, APVAC2, was constructed to target neoantigens. The rationale behind the use of two vaccines was to combine both tailored antigen landscapes into the patient's standard care to improve the immunotherapeutic potential for more efficacious treatment of the 15 newly diagnosed patients. The vaccines were administered with adjuvant immunostimulants and were seen to induce $CD8^+$ and $CD4^+$ T-cell responses for APVAC1 and 2, respectively, with an impressive median overall survival of 29.0 months and a median progression-free survival of 14.2 months.

The integration and analysis of data derived from multiple omics platforms necessitate robust bioinformatic tools and specialized expertise [201], and the functional validation of neoantigens necessitates further immunological assays to confirm their ability to elicit a potent T-cell response. Despite these hurdles, multi-omics approaches present a powerful and promising strategy for unraveling the complexities of the GBM proteome and identifying meaningful target proteins for the development of transformative immunotherapies.

# Methods

The main methodologies used in this thesis are described below. See the Material and Methods sections in **Papers I-IV** for further information.

## Cell culture

The cultivation of simplified models of biological tissues is a technique first developed in 1907 when Ross Harrison extracted nerve tissue from frog embryos to closely study their development [202]. While these nerve tissues could only be kept alive for a maximum of four weeks at the time, modern approaches have widely improved cultivation methods to the point that immortalized cultures are possible today. In general, the cultivation of isolated cell populations is a low-cost tool extensively utilized in research that allows for the understanding of metabolism, morphology, cell biology, drug action, and many other processes [203]. By growing a cell subpopulation in a controlled two or three-dimensional cell culture, the complexity of the sample decreases and specific phenomena can be studied while isolated from other influencing factors that would otherwise, add noise and hinder the basic understanding of a cellular event of interest.

### *Two-dimensional monolayer cultures*

Throughout **Papers I-IV**, several monolayer cell culture models representative of GBM, endothelial, and immune cells were utilized to understand the effects of the TME in GBM. In **Papers I**, **III**, and **IV**, the U87MG cell line was used, which is the most studied GBM 2D model to date ( >9000 PubMed results for U87MG and its name variations). While this cell line has been instrumental in advancing the understanding of GBM since its establishment in the late 60s [204], long-term cultivation has probably culminated in the loss of its original phenotype, making it less representative of the original tumor [205]. Moreover, the misidentification of other GBM cell lines caused samples of unknown origins to be designated as U87MG [206]. Upon a thorough analysis by Allen *et al.*, despite the unknown origin, the commercially available "U87MG" cell line was confirmed to have CNS origins and to be a genuine human GBM model [206].

As assessed in **Paper III**, the transcriptional profile of the U87MG cells used in the studies presented within this thesis is that of a mesenchymal GBM and analysis of the culture's genetic makeover, according to COSMIC cell line gene mutation database (ID COSS687590), shows it carries classical GBM traits such as PTEN and NF1 mutations and wildtype IDH gene [207–209]. Nowadays, the use of U87MG cells in academic experiments should be carefully considered and, if utilized, further steps such as the inclusion of additional GBM models in the same study should be taken to minimize the risk of biased conclusions. Therefore, in **Papers I**, **III**, and **IV**, further GBM models ranging from patient tumor cells, primary cell lines, or mouse models were added to the study design.

In **Papers II-IV**, the following primary GBM cell lines were used: U3065MG, U3082-MG, U3034MG, U3047MG, U3017MG, and U3054MG. These cells, which were recently isolated from patient tumors and established into primary GBM cultures by the Human Glioblastoma Cell Culture (HGCC, www.hgcc.se) resource [210], underwent a lower amount of expansion steps and thus, have lesser subclonal selectivity than the immortalized U87MG cells. The cultivation of these primary cells in serum-free media allows them to retain more of the genomic and transcriptomic characteristics found in their tissue of origin [211, 212], thus being more reliable in reflecting tumor biology and becoming a key model for brain tumor research. However, differently than immortalized cell lines, primary cell lines have a limited lifespan and undergo senescence after a limited amount of passages [211, 212]. Moreover, the expansion of primary cultures has to be well controlled and cared for, as prolonged expansion was seen to lead to transcriptional drift and changes to the cell's clonal composition [213].

To study the influence of extracellular lipids on immune cells and ECs, primary Human Brain Microvascular Endothelial Cells (HBMECs) and the THP1 cell line were employed in **Papers I** and **IV**. HBMECs are suitable for studies on the behavior of brain ECs, including the BBB [214–216], and THP1 is a human leukemic monocyte cell line which can be differentiated into macrophages [217, 218]. Lastly, in **Paper I**, the GL261 cell line was used, which is one of the most frequently used murine GBM models, initially established in 1970 [219, 220]. These cells were propagated as monolayer cell cultures but also utilized for mouse injections for the development of GBM mouse models (see **Mouse models** below).

### *Three-dimensional cultures*

To better understand the spatial distribution of protein expression in GBM, spheroids were generated in **Papers II** and **III**. These three-dimensional structures offer several advantages over adherent and semi-adherent 2D cultured cells [221, 222]. For once, the organization of cells into clusters allows this model to better mimic the tumoral tissue

architecture, granting multilateral cell-to-cell interactions and the formation of oxygen and nutrient gradients, which naturally occurs in a growing *in vivo* tumor [222, 223]. Such gradient diffusion enables the emergence of cell subpopulations, where cells near the borders of the spheroid are fully nourished and actively participate in tumor expansion, while cells closer to the core of the spheroid experience limited access to nutrients and oxygen, triggering cell adaptive responses, and leading to a more aggressive phenotype seen to contribute to therapeutic resistance [221–224]. Thus, spheroids offer a more complex range of cell types that can present with differential protein abundances, as seen in **Paper III**, and that respond differently to treatment studies, as seen in **Paper II**.

The disadvantages of working with 3D cultures fall under the same reason that makes it more realistic: its complexity. Spheroid cultures can be unsuitable for initial explorations as the outcomes are influenced by several factors (cell subpopulation, cell interactions, nutrient gradient, differences in pH, etc.), thus not enabling a more direct and isolated study of the association between a cause and its effect that a simplified 2D setup allows. Moreover, 3D cultures are technically more demanding and need rigorous standardization (*e.g.* optimizations in seeding cell density and homogenization of final spheroid size) to increase experiment reproducibility by reducing the variability in spheroid size and morphology. In addition, 3D cultures require longer incubation periods before spheroids are ready to be utilized in experiments.

## Mouse models

Mice (*Mus* sp.) are currently the primary model organism employed for *in vivo* cancer research, serving as a bridge between cell-based and clinical research. Mouse models enable a comprehensive investigation of tumor development, progression, and therapeutic response within a whole-organism framework and in a cost-efficient manner [225, 226]. The effectiveness of this model lies in its ability to replicate the interactions between tumor cells, the immune system (if desired), and the surrounding environment, which are largely limited by *in vitro* approaches. Additionally, mice share considerable genetic similarity to the human genome and exhibit short life cycles, both of which are highly desirable traits for research applications [226]. The development of advanced *in vivo* models (*e.g.*, genetic knock-in or knock-out specimens) has facilitated the creation of tailored solutions for a diverse range of research needs, including the study of specific gene contributions, the enhanced recapitulation of human tumor biology, and more efficient prediction of potential clinical outcomes. Among the different types of animal tumor models [226], two variations of transplantable models were used in **Papers I** and **IV**: Syngeneic/allogeneic and xenogeneic, which are briefly described below. All experimental procedures with *in vivo* models were approved by the ethical committee

for animal research in Malmö/Lund and were in accordance with the European Union directives.

### *Syngeneic and allogeneic*

In syngeneic mouse models, mice are engrafted with tumor cells derived from genetically identical mouse strains. Thus, in **Paper I**, C57BL/6 mice (ApoE knock-out or wildtype) were injected with GL261 tumor cells, a GBM cell line created in C57BL/6 mice via carcinogen induction [219]. Tumors derived from injected GL261 cells were seen to preserve several similarities to human GBMs, such as anaplasia, high mitotic activity, and even a representation of the pseudopalisading necrosis that frequently occurs in patient-derived GBMs [227] but also presented diverging features such as activating Kras mutation that is rarely seen to happen in human GBMs [228]. In this model, since the tumor is derived from the same species as the recipient animal, the absence of graft rejection to the transplanted cells enables it to reflect the natural behavior of tumors more accurately, and thus, immunocompetent mice can be utilized. On the downside, GL261-generated tumors are highly immunogenic due to an intrinsic high mutational burden, which prompts it to have an increased MHC-I antigen presentation than human GBMs [227]. Moreover, the hypermutated cells present a higher sensitivity to therapeutic interventions, such as radiation therapy, and their use for assessing treatments might not be optimal [220].

In allogeneic mouse models, mice receive tumor cells from non-identical strains, thus bearing a different genetic background and requiring the use of immunodeficient mice to avoid rejection of the material. Thus in this same study, allogeneic models were created by injection of GL261 cells into Non-Obese Diabetic (NOD)/Severe Combined ImmunoDeficiency (SCID) mice.

In **Paper I**, syngeneic and allogeneic mice were employed to study the extracellular lipids as a driving stress-adaptive mechanism of GBM cells influencing the modulation of ECs, angiogenesis promotion, and macrophage infiltration within the hypoxic microenvironment. The induction of the lipid-loaded phenotype was achieved by either injecting tumor cells primed with lipids (LD+) into the brains of either immunocompromised (NOD/SCID) or immunocompetent C57BL/6 mice, or by feeding immunocompetent Apolipoprotein E knock-out (ApoE-/-) mice with a high-fat diet (21% fat) 4 weeks before glioma cell injection. In the initial *in vivo* experiment, NOD/SCID mice were injected with hypoxic GL261 cells pre-conditioned with LD+ or LD-, and tumor size, survival, macrophage infiltration (which are still present in immunocompromised mice), and angiogenesis were assessed. Next, immunocompetent mice were utilized to further understand how hyperlipidemia regulates these processes. Thus, C57BL/6 mice, either wildtype on a chow diet or ApoE-/- knock-out mice on high-fat diet were

orthotopically injected with primed GL261 cells and survival, macrophage infiltration, and angiogenesis were assessed.

### *Xenogeneic*

In xenogeneic mouse models, cells of a different species are introduced into mice and, due to immunological incompatibilities, the immune system of the host animal has to be suppressed to decrease rejection of transplanted tissues or cells. When human cells are implanted in mice, the model is also known as a xenograft [229]. One of the most commonly used immunodeficient mouse strains is the SCID mouse (severe combined immunodeficiency), which was first reported in 1983 [230] and lacks mature T and B lymphocytes, the primary cells responsible for adaptive immunity, due to a point mutation in the Prkdc gene [226, 231]. The features of this strain were further combined with additional modifications to generate novel strains with enhanced ability of human cell acceptance, such as SCID/non-obese diabetes (NOD) strain that presents with the absence of circulating complement, and the NOD/SCID gamma (NSG) mice, a strain bearing a mutated IL2 receptor gamma chain ($IL2rg^{null}$) to prevent cytokine-mediated "leaking" immune signaling via several receptors, culminating in a NK cell deficiency, extreme immunodeficiency, and a higher rate of human-cell implantation [229, 231]. Currently, NSG mice are the gold standard for this category of *in vivo* model, valuable for studying human transplantation, tumor growth, metastasis, and the effects of CAR-T-based immunotherapies [226]. The disadvantage of xenogeneic models is the handling of immunocompromised mice, which require a sterilized environment, and the fact that the absent immune cells, if not exogenously provided, are important players in tumor biology that will not be accounted for within the model system [229].

In **Paper IV**, NSG mice were co-injected with $2x10^5$ U3054MG cells primed with $4x10^5$ differentiated hypoxic macrophages pre-treated with EVs (50ug/mL) and with or without Triacsin C (*i.e.* an upstream inhibitor of DGAT1, which precludes LD formation by GBM cells) for 24 hours for the study of tumor-associated foamy macrophages contribution to tumor formation and angiogenesis. After 4 weeks from the initial injection, the tumors were replenished of exogenous macrophages in a second round of injections. Additionally, non-treated cells from the U3054MG and U3047MG cell lines ($3x10^5$) were injected into NSG mice to evaluate how metabolic stimulation (ATP levels) by GBM conditioned medium and EVs depend on LD formation. Mice from both groups were simultaneously sacrificed when the first mice displayed symptoms of neurological distress and the dissected tumors or normal brain tissues were cryopreserved or digested into single-cell suspensions for further experiments.

## Patient-derived specimens

Patient tumor tissue specimens are an important resource for the discovery of clinically relevant results. These specimens offer a direct window into the different aspects of human biology, allowing for the validation of several research hypotheses. Throughout **Papers I - IV**, GBM samples collected from consenting patients (18 years of age or older) referred to the Department of Neurosurgery at Lund University Hospital were utilized. All studies were carried out according to the international ICH-GPC guidelines [232], the Helsinki Declaration [233], and approved by Lund University Regional Ethical Board.

### *Brain tumor specimens*

GBM tissue samples were obtained from surgical biopsies performed at Skåne University Hospital. In brief, patients with suspected glioma were diagnosed through routine brain MRI scans, followed by surgical intervention and pathological classification. Next, standard oncological treatment was given and patients received follow-up care according to national guidelines. The tumor specimens obtained (see example in Figure 8A) (WHO grade II to IV) were either directly processed (for the establishment of single-cell suspensions or TS-MAP protocol) or snap-frozen with isopentane and stored at -80°C. For the establishment of single-cell suspension from fresh tissues, the tumor bulk was fragmented with a scalpel and underwent enzymatic dissociation with TrypLE Express and DNase I for 10-20 minutes at 37°C on an orbital shaker. Digested tissue was then filtered through 70 and 40 µm nylon cell strainers, followed by red blood cell removal with red blood cell lysis buffer for the establishment of patient-derived cultures, and the culture cells were seeded in culture media according to the requirements of each experiment (Figure 8B).

### *Tumor cryosections and Laser capture microdissection*

The advantage of snap-freezing tumor specimens lies in preserving their cellular architecture for future microscopic examination. Once mounted in a support frame, frozen GBM tumors were sectioned into 10 µm thick slices and carefully placed on top of DNase and RNase-free membranes, following cell nuclei staining with cresyl violet and preparation for Laser capture microdissection (LCM). LCM, a technique first proposed in 1986 [234] and suitable for the separation and collection of specific areas within a mounted tissue section, was used in **Papers I** and **IV** to enable the profiling of cancer cells or macrophage-infiltrated tumor regions, respectively, with or without lipid loaded phenotype (LD+ and LD-) (Figure 8C).

Following membrane mounting, samples underwent dehydration in cold ethanol to reduce hydrostatic forces, and adjacent sections used for the identification of the regions of interest (ROI) were mounted on glass slides and stained for nuclei with Hoechst 33342, CD68 as macrophage marker, and HCS LipiTOX for distinguishing LD+ and LD- areas. For tissue microdissection, the Zeiss' PALM LCM system was utilized, which implements a UV laser to dissect the corresponding ROI of the membrane-mounted tissue after initial ROI identification within the reference adjacent slide. For sample collection, the system applies a UV laser pressure to push the sample piece into the collection tube, minimizing sample manipulation and mechanical damage. This system allows for quick and efficient sample collection but, in turn, can damage the mRNA at the immediate border of the cut sample, thus limiting the minimal size of the cutout tissue for the sake of mRNA quality [235]. Dissected LD+ and LD- regions of tumor cells (Hoeschst$^{+}$, CD68$^{-}$, **Paper I**) or macrophage-loaded areas (Hoechst$^{+}$, CD68$^{+}$, **Paper IV**) were then profiled with the Affymetrix Clariom D Pico Gene Array.

***Blood samples***

In **Papers I** and **IV**, blood samples from baseline patients (pre-operatively) and voluntary healthy donors were collected into EDTA-containing tubes, centrifuged at 2000x g for 10 minutes at room temperature, and either stored at -80°C until further preparation or freshly used. For the first paper, the plasma of patients from the "MRI study" cohort was analyzed for the presence of tumor-associated markers and, for **Paper IV**, blood was collected in association with the Clinical Immunology and transfusion division for the isolation of peripheral blood mononuclear cells (PBMCs) (Figure 8D).

In **Paper IV**, PBMCs were readily collected from the buffy coat of patient blood samples after density-gradient centrifugation and monocytes were further isolated from the PBMC mixture by positive selection of CD14$^{+}$ marker via the use of antibody-coated microbeads according to the manufacturer's instructions. Isolated monocytes were then cultured (1x10$^{6}$ cells/ml) in µ-glass bottom slides (8 or 16 wells) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% L-Glut, 1% Penicillin-Streptomycin and 15 ng/ml of macrophage colony-stimulating factor (M-CSF). For the differentiation into primary macrophages, cultured cells had their medium replaced with fresh serum-free medium, and M-CSF was added accordingly during days 3 to 7. On the 6th day, granulocyte M-CSF (GM-CSF) was added to the cell medium for improved cell viability (Figure 8D). For the differentiation of THP-1 cells into a macrophage-like state, cultured cells were induced with 50 nM of PMA for 48 hours, followed by recovery in serum-free medium without PMA for 48 hours.

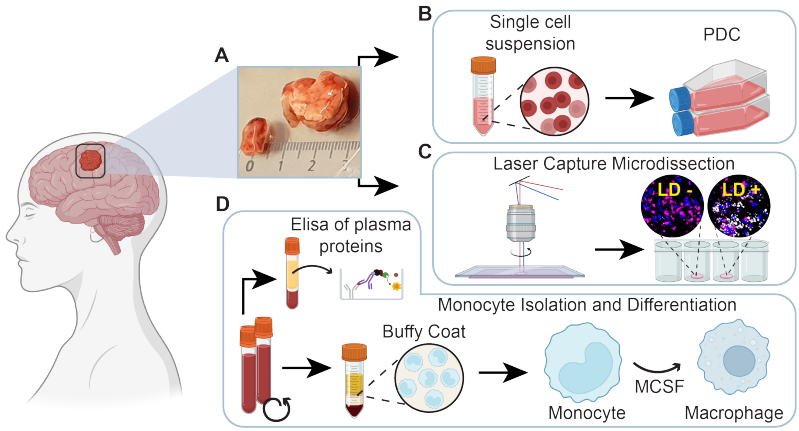


**Figure 8. Patient-derived sample processing.**
**A)** Example of fresh tumor sample (ruler scale in centimeters). **B)** Generation of Patient-Derived Cultures (PDCs) from a single-cell suspension of tumor samples. **C)** Laser capture microdissection of LD loaded or LD scarce areas. **D)** Processing of samples for plasma protein assessment or for the isolation of monocytes from the buffy coat of Ficoll centrifuged blood, followed by macrophage differentiation. Made with BioRender.

## Hypoxia induction

Hypoxia is one of the main underlying TME features of GBM, and its effects were studied throughout **Papers I**, **III**, and **IV**. The incubation of 2D models at 1% of $O_2$ was used to mimic the hypoxic environment found within the core of GBM tumors. The 1% oxygen level was seen to provide a good experimental balance between HIF-1 stabilization and cell expansion rate since GBM models significantly reduce proliferation when conditioned to hypoxia [90]. Hypoxic conditioning was achieved by incubating cells in a SCI-tive N-N hypoxia station (Baker Ruskin) set at 1% $O_2$, 5% $CO_2$, 94% $N_2$, 80% humidity, and 37°C at specified durations.

## Isolation and characterization of Extracellular Vesicles

Isolation of EVs was performed in **Paper IV** for the assessment of EV-derived lipids on GBM TME. In this study, EVs were obtained from collected media of hypoxic U87MG GBM cells cultured in serum-free DMEM supplemented with 1% Bovine serum albumin or from media of hypoxic primary cultures grown in Neurobasal medium supplemented with 1% N2 and 1% Penicilin-Streptomycin. The use of serum-free media before EV isolation is crucial to exclude contamination with EVs and lipoproteins present in serum-containing media.

After collection, the conditioned medium was centrifuged twice at 400 x g at 4°C to remove remaining cellular debris, followed by EV pelleting by ultracentrifugation, which is a commonly used method and involves centrifuging the supernatant at different speeds to separate vesicles according to their size and density. In the first centrifugation round, the supernatant was spun at 100,000 × g at 4°C for 2 hours, following a pellet wash (with phosphate-buffered saline) and two additional ultracentrifugation rounds at 100,000 × g for 2 hours. Although efficient, ultracentrifugation is a time-consuming approach and may result in sample loss due to aggregation of EV particles.

To characterize the EV particles in **Paper IV**, the ZetaView (Particle Metrix) and the Exoid (Izon) were used for high-resolution measurement of particle size and concentration. ZetaView utilizes dynamic light scattering (DLS) to perform nanoparticle tracking analysis (NTA), measuring EV size distributions based on the scattered light pattern captured by a digital camera over a series of frames after EVs are illuminated by a laser. When compared with other measurement methods such as the NanoSight NS300, the ZetaView was observed to be an accurate tracker system but sensitive to particle aggregation, which may lead to underestimation of the true size of EVs [236]. On the other hand, the Exoid uses tunable resistive pulse sensing (TRPS) to measure the concentration, size, and zeta potential of particles within a solution, which outperforms DLS-based NTA for particles above the 150 nm size and can detect more particles than NTA [237]. In TRPS, EVs are passed through a pore sensor, changing their electrical resistance as they pass through and resulting in a resistive pulse signal. However, stable pore aperture and stabilization of the pore current, both required before sample measuring, have proven to be challenging to achieve. After sample characterization, the average sizes of U87MG or U3017MG derived EVs were seen to peak around 120 nm with a size range from 100 to 250 nm, while EVs of conditioned medium from patient-derived cultures peaked at around 100 nm and ranged from 50 to 150 nm. Western blotting of isolated Evs displayed exosome-like characteristics, which comprised the presence of CD63 and CD9 proteins in EV samples, as well as the absence of EEA1 protein.

## Gene expression quantification

Fluctuations in gene expression levels can be effortlessly measured nowadays with varying degrees of resolution and throughput thanks to a diverse range of commercially available techniques for transcriptomic profiling. Of these, high-throughput approaches, which measure thousands of transcripts in one run, include microarrays and RNA sequencing (RNA-seq). Microarrays determine the gene expression abundance through the fluorescence intensity emitted upon the hybridization of the fluorescently labeled complementary DNA (cDNA) sample with the short predetermined set of nu-

cleotide oligomer sequences (the probes) tethered to a solid array base (the chip) [238]. Used in **Papers I** and **III**, the HumanHT-12v4 Expression Illumina BeadChip (Illumina) microarray requires around 750 ng of sample and hybridize transcripts onto probe-coated beads (approx. 47000+ probes in total) distributed across a chip [239], thus being chosen in these studies for the profiling of abundant cell line samples. On the other hand, when sample amount was a limitation, the Clariom D Pico (Affymetrix) array was used in **Papers I** and **IV**. This microarray platform utilizes multiple different short probes (>4x$10^6$ exon-targeting probes) directly distributed across the chip and only requires between 100 pg to 50 ng of isolated RNA [240].

For **Papers III** and **IV**, RNA-seq was applied for the assessment of total RNA or single-nuclei RNA, respectively. In RNA-seq approaches, upon mRNA fragment conversion into cDNA, adapter sequences are inserted. Contrary to microarray, which uses predetermined probes that hybridize directly with the transcript sequence, RNA-seq flowcells present with covalently bound oligos that hybridize with the cDNA adapters, acting as primers for bridge PCR amplification. After the formation of clusters of amplified fragments, high-throughput sequencing is initiated using Sequencing by Synthesis technology. This process involves the sequential addition of fluorescently labeled nucleotides, each emitting a unique light signal upon incorporation into the growing complementary DNA strand. The emitted light is then detected, enabling the determination of the nucleotide sequence being synthesized [241]. When compared to microarrays, RNA-seq has become the preferred approach for gene expression quantification due to its high resolution, lower technical variation, and expanded data application (*e.g.* study of novel transcripts) [242].

### Tumor surfaceome mapping

Tumor surfaceome mapping (TS-MAP) is a new platform initially developed by Guilmain *et al.* [243] and further developed by Governa *et al.* [244] to comprehensively characterize the protein landscape on the surface of tumor cells. This approach was created to aid cancer immunotherapy, where the identification of surface proteins comprises a bottleneck for therapeutic development, and the characterization of internalizing surface proteins within a given sample plays a crucial role in predicting the efficacy of some immunotherapy modalities. TS-MAP leverages a combination of intact sample biotinylation, mass spectrometry, and bioinformatic analysis to identify and quantify surface proteins from intact patient-derived tumor samples, mouse tumors, and primary 3D and 2D cultured cells. This profiling methodology, utilized in **Papers II** and **III**, facilitates the identification of potential therapeutic targets, particularly for ADCs and CAR-T cells.

In the TS-MAP protocol, alkalized samples undergo incubation with a reversible bi-

otin tag (Figure 9). The utilized biotin contains a Sulfo N-hydroxysuccinimide ester that forms stable bonds with available primary side chain amines of extracellular lysine residues or N-termini of polypeptides when in alkaline conditions (pH 8.0). The alkaline incubation enables lysine amines to be in their unprotonated state and ready to react with the tag [245]. Due to its negative charge, the biotin reagent does not permeate the cell membrane, being optimal for the exclusive tagging of surface proteins. After the removal of the biotin solution, free biotin is quenched with glycine, which sequesters the remaining reagent with its primary amines. At this stage, surface proteins are ready to be obtained (Figure 10A, Surface Target), *a.k.a.* the surfaceome, or samples can undergo incubation in endocytosis permissive conditions to allow for the internalization of labeled proteins once uptake is resumed, to retrieve the endocytome (Figure 10A, Endocytosed Target). Samples directed for the capture of endocytosed biotinylated proteins then undergo removal of remaining surface biotin via reduction of the disulfide bond by incubation with the reducing agent sodium 2-mercaptoethanesulfonate (MesNa) (Figure 9B), then Iodoacetamide alkylation of the remaining spacer fragment bound to surface proteins, and further quenching of possible free biotin via incubation with unconjugated streptavidin (50 µg/ml, 30 min, 4°C). Once both fractions, surfaceome and endocytome, are prepared, samples can be directed to flow cytometry or confocal microscopy using streptavidin-conjugated fluorophores, western blotting us-

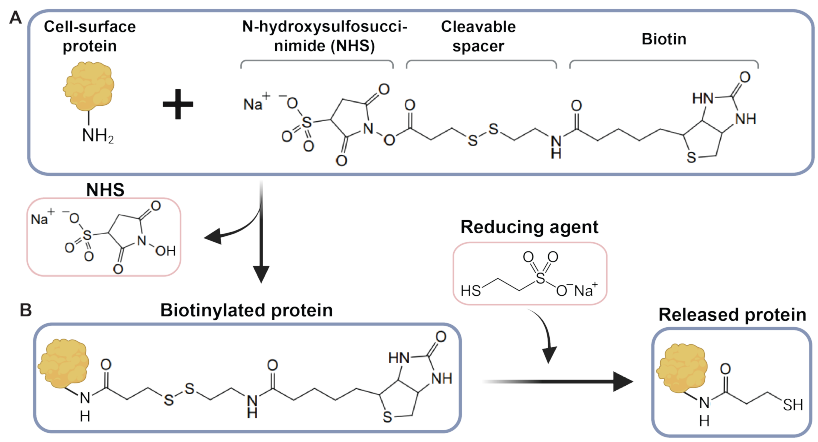


**Figure 9. Reversible protein biotinylation.**
**(A)** Exposed primary amine groups of proteins react with the N-hydroxysulfosuccinimide (NHS) group within the EZ-Link Sulfo-NHS-SS-Biotin reagent under alkaline conditions, releasing it as a byproduct. **(B)** The biotin molecule enables the purification of tagged proteins via immobilization of the sample in streptavidin HPLC column thanks to the strong interactions between biotin and avidins (not represented in figure). The longer spacer of the reagent structure reduces steric hindrances associated with avidin binding. Moreover, after the washing of non-labeled proteins, biotinylated proteins can be released by reducing the disulfide bond within the spacer with reducer agents such as MesNa. Inspired by [245, 246]. Made with BioRender.

ing a streptavidin-HRP antibody, or they can undergo affinity column purification for further mass-spectrometry characterization (Figure 10B).

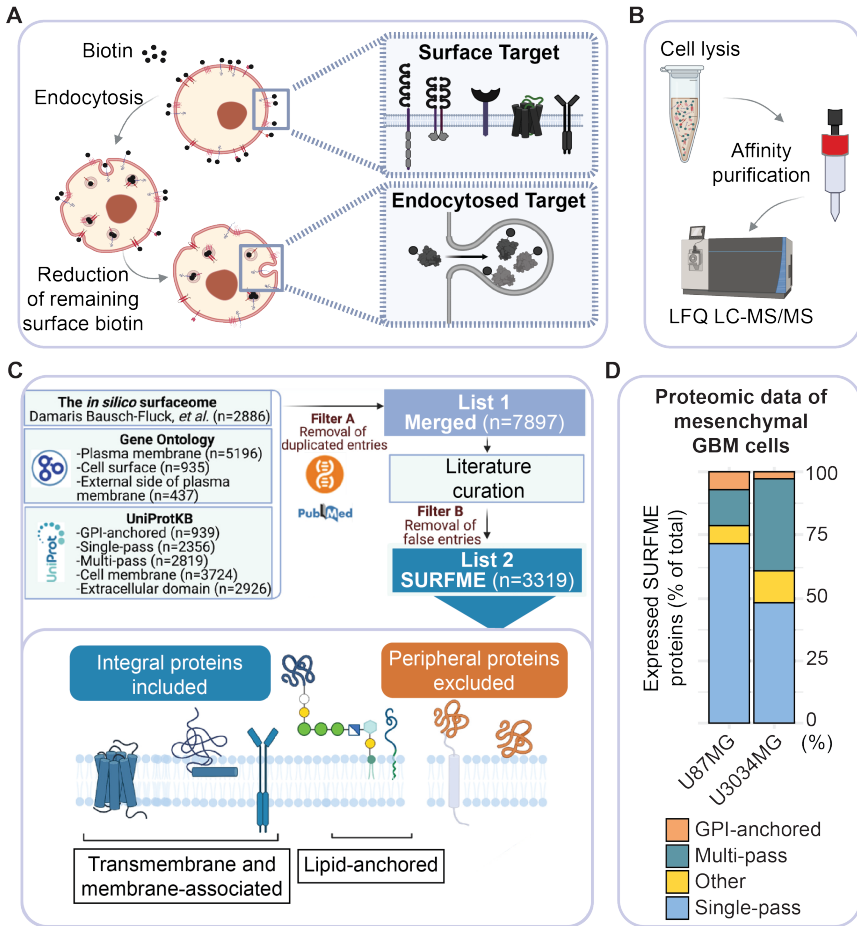


**Figure 10. TS-MAP workflow.**
**(A)** Cells are incubated with biotin for retrieval of surface and endocytosed targets. **(B)** Biotinylated cells are lysed, purified, and peptide samples are characterized for label-free mass-spectrometry (LFQ LC-MS/MS). **(C)** Steps for building the SURFME filter: Hits in the human *in silico* surfaceome were merged with protein accessions obtained from Gene Ontology and UniProtKB terms such as "External side of plasma membrane" and "Extracellular domain". After curation and list updating, the SURFME filter comprises a set of 3319 accessions of membrane-related proteins. **(D)** Example of the categories overrepresentation. Proteomic characterization of two mesenchymal GBM models (U87MG and U3034MG) followed by SURFME filtering allows for visualization of differences in surface repertoire: U87MG, a cell line that displays heavy clonal selection, shows a striking overrepresentation of single-pass transmembrane proteins while the primary cell line, U3034MG, presents with a lower representation of the same category and higher percentage of multi-pass proteins. Modified with permission from Governa *et al.* [244] and de Oliveira *et al.*.

Notably, the TS-MAP integrates with the SURFME classifier (Figure 10C), an in-house curated resource specifically designed to filter mass-spectrometry results for *bona fide* surfaceome proteins and to provide annotation for protein category over-representation assessment (see example in Figure 10D). The SURFME resource concatenates the set of 2886 proteins predicted to belong to the human surfaceome by Bausch-Fluck *et al.* [247] and 400+ other proteins known to be membrane-associated (*e.g.* CA9 [248]), obtained after mining and curation of known public databases such as Uniprot and Gene ontology. The integration of proteomics data with SURFME enables researchers to efficiently prioritize targets based on their structural features and to focus complex proteomics results for the set of membrane-associated proteins.

## Mass-spectrometry

As previously described in the section **Proteomics tools**, mass-spectrometry is a widely used technique for the analysis of complex peptide mixtures. Thus, label-free quantitation high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) was applied in **Papers II** and **III** for the identification and quantification of isolated surfaceome and endocytome proteins of bulk tumors and cultured cells. For these studies, the Fusion Tribid mass spectrometer was utilized, which combines the Orbitrap quadrupole with a linear ion trap mass spectrometer for parallelization of data acquisition (while the Orbitrap performs high-resolution quantitation, the Ion Trap detection does lower-resolution readouts), enabling more data to be collected at the same time for increased identification rates of high and low abundance peptides [249].

During sample preparation, isolated and purified surfaceome/ endocytome proteins were resuspended in urea 6 M buffer, reduced with 10 mM dithiothreitol for disruption of disulfide bonds, and alkylated with 50 mM Iodoacetamide to cap the reduced cysteine residues. After pH adjustment, protein samples were digested by trypsin, and desalted peptides were dried and kept at -20 °C short term until concentration adjustment. Before LC-MS/MS analysis, samples were resuspended in Acetonitrile and Trifluoroacetic acid, quantified, and adjusted to a final concentration of 0.5 µg/µL. Then, solutions were centrifuged and transferred into mass-spectrometer vials, which were stored at -20 °C until instrument loading. Prior to sample injection, peptide samples were first concentrated on-line on an Acclaim PepMap 100 C18 (75 µm x 2 cm) pre-column, following separation on an Acclaim PepMap RSLC (75 µm x 25 cm, nanoViper, C18, 2 µm, 100 Å) column with a solvent gradient to generate a non-linear peptide elution. In total, 1 µg of peptides were introduced into the instrument (in triplicate injections) via nanoelectrospray ionization with the Nano-bore emitter (OD 150 µm, ID 30 µm). The spray voltage was set at 2 kV and capillary temperature at 275 °C for generation of the gas-phase ions. The orbitrap detector first measured all intact peptide ions through

full MS survey scans between m/z range of 350 to 1350 and a resolution of 120,000. To enable the ions to enter the mass analyzer at specific times, automatic gain control target was set to 4 x $10^5$ with a time of 50 ms. Up to 20 most intense ions from the full scan MS with charge states 2-5 were selected for further fragmentation in the Orbitrap, precursors (MS2) were isolated on the quadrupole mass filter set to 1.2 m/z of width and underwent high-energy collision dissociation fragmentation at 30% of normalized collision energy. Resolution was fixed at 30,000 and for MS/MS scans, automatic gain control target value and injection time were $5 \times 10^4$ and 54 ms, respectively. Dynamic exclusion duration, *i.e.* the period a spectrum is prevented from being further fragmented [250], was set to 45 s and the peptide mass tolerance window was 10 ppm to allow for specific, but not too stringent, tandem mass spectrum comparison [251].

For data analysis, raw files were input into the Proteome Discoverer 2.3 software, and spectra were searched for peptide identification with SEQUEST HT search engine equipped with UniprotKB human database. The peptide search was performed with the modifications: cysteine carbamidomethylation (resulting from cysteine alkylation [246]) as static modification, and both methionine oxidation (which commonly accumulates during trypsin digestion and electrospray ionization [252]) and N-terminal acetylation (an abundant irreversible PTM [253, 254]) as dynamic modifications. Precursor tolerance was 10 ppm, fragment tolerance was set to 0.05 Da, up to 2 missed cleavages were allowed, and peptide validation was done with Percolator with a maximum q-value of 0.05.

## Differential expression analysis

Data generated by high-throughput technologies such as RNA-seq, mRNA microarray, and proteomics result in thousands of identified genes or proteins measured across several samples. To efficiently mine the data generated by these platforms and uncover the biological mechanisms underlying a condition, Differential Expression Analysis (DEA) is often employed. This approach takes the normalized abundance measurements for genes or proteins (*e.g.* count matrix of RNA-seq data) and performs ratios between the abundances of compared groups (*e.g.* mean expression of gene A in tumoral samples divided by the mean expression of gene A in healthy samples), following statistical analysis to reveal the significance of the obtained quantitative modulations between experimental groups.

For RNA-seq-based approaches, the statistical calculation for differentially expressed genes (DEGs) is often based on negative binomial distributions, which take into consideration that more variations in gene expression are observed when analyzing multiple samples [255], and tools such as EdgeR [256] and DESeq2 [257] are reliable to perform this task. However, in intensity-based quantifications, such as proteomics or microarray

data, the DEA is based on a combination of linear model statistics, and the tool limma [258] can thus be employed. In limma, a contrast matrix is used to specify the relationship between the gene expression and the experimental factors (*e.g.* which samples are the reference/control) for the design of a linear regression model for the estimation of the effect of each factor on gene expression. Next, t-test is usually performed for the significance assessment of the drawn ratio.

### Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether a pathway signature displays a statistically significant difference between two biological states and it was applied in **Papers II-IV**. This tool is particularly useful to observe whether certain pathways are affected by a condition in comparison to its control sample (*e.g.* pathways enriched in tumoral versus healthy samples or hypoxic versus normoxic cells) [259].

Instead of focusing on the assessment of individual genes, GSEA evaluates predefined sets of genes (known as genesets), considered to be representative of the activation or repression of a biological function or metabolic pathway. These genesets are sourced from databases such as the Molecular Signatures Database, the Kyoto Encyclopedia of Genes and Genomes, Reactome, Gene Ontology, and other sources. Once the expression values of samples and the genesets are input into the GSEA software, it then assesses whether genes from these sets are statistically over-represented among differentially expressed genes between experimental groups, facilitating the identification of biological pathways or processes underlying a phenotype of interest and extracting meaningful biological insights from complex datasets. Since the GSEA algorithm utilizes a ranking-based approach and running-sum statistics, it can be applied to transcriptomic, metabolomic, proteomic, and any other high-throughput dataset with normalized values, and such versatility is of great use for the capture of underlying biological pathways for multi-omics integrated data [260].

### Immunoprofiling

Immunoprofiling represents a myriad of techniques employing antibodies to quantitatively assess protein abundance or distribution in complex biological samples. This approach benefits from the specificity of antibodies toward their respective epitopes for sensitive detection of target proteins, and comprises methods such as enzyme-linked immunosorbent assays (ELISA), multiplex immunoassays, flow cytometry, and immunofluorescence, each offering unique strengths and limitations. Regardless of the

chosen method, these techniques rely on the selection of high-quality, validated antibodies, as their specificity and efficacy directly impact the accuracy and reliability of data generated. Some of the immunoprofiling methods utilized throughout **Papers I-IV** are described below.

### *ELISA*

Enzyme-linked immunosorbent assay (ELISA), a method developed by Engvall and Perlmann in 1971 [261], serves as a robust and widely employed technique for singleplex quantification of protein abundance in biological samples such as supernatants, serum, and plasma, and it was implemented in **Papers I** and **IV** for measuring the abundance of HGF and VEGF-A proteins in conditioned medium. The ELISA assays utilized in these papers employed the quantitative sandwich enzyme immunoassay technique. This format involves incubating the human sample in microplate wells precoated with antibodies specifically binding the target protein. Next, once the protein of interest is bound, the sample is incubated with another solution of target-specific antibody either labeled directly with an enzyme or labeled with biotin, forming a “sandwich" configuration. In the case of the biotin-labeled antibody, streptavidin-HRP is then added to covalently bind to the available biotin. Lastly, a chromogenic substrate solution reactive with the employed enzyme is added to the wells, resulting in a colorimetric signal upon enzymatic conversion. Once the reaction is stopped, the optical density is measured in a plate reader at 450 nm and the absorbance intensity positively correlates with the amount of captured protein, enabling estimation of protein abundance via comparison of the sample signal to a set of standard dilutions of known concentrations.

### *Proximity extension assay*

Unlike ELISA, PEA is a multiplexed and high-throughput immunoassay [262]. In **Paper I**, PEA was implemented for the quantification of secreted proteins in the conditioned medium of GBM cell cultures and in the plasma of GBM patients or healthy subjects. For this purpose, the panels Proseek Multiplex Oncology II and CVD III panels from Olink Bioscience were utilized. In **Paper IV**, conditioned medium from primary macrophages exposed to serum-free medium supplemented or not with GBM-derived extracellular vesicles for 24 h in hypoxia was profiled with PEA, and proteins were analyzed using the Proseek Multiplex Onco-Immunology Olink panel.

The PEA technology benefits from the use of DNA-tagged specific antibodies. Once a pair of oligonucleotide-labeled antibodies bind to their target protein, the proxim-

ity allows for hybridization of their tags, generating detectable amplicons that are then PCR-amplified and quantified on Illumina NovaSeq instruments [262]. This unique approach benefits from key steps such as the exclusive hybridization of correctly matched DNA reporter pairs, which prevents false positives by elimination of cross-reactive events, and; the enzymatic amplification of the reporter sequences, which generates detectable DNA products proportional to the initial protein levels. The employment of DNA sequences as reporters for protein abundance enables the sensitive and multiplexed quantification of numerous proteins simultaneously since specific barcode sequences can be generated for the identification of each protein [262]. Compared to conventional methods, PEA displays reduced background noise, higher sensitivity, and the ability to analyze large numbers of proteins in a single experiment [262, 263].

### *Fluorescence microscopy*

Fluorescence microscopy is a methodology widely utilized throughout **Papers I-IV** for the detection and subcellular structure localization of target proteins. This approach relies on the use of fluorophores (*i.e.* molecules that absorb light of a specific wavelength and emit light of a longer wavelength) for the staining of biological samples such as cultured cells or tissue sections. The fluorescent compounds can be conjugated to antibodies for the specific staining of proteins of interest or can present as free dyes, which is the case for the Hoescht dye commonly utilized for the counterstaining of cellular nuclei.

The preparation of samples meant for similar modalities of fluorescence microscopy follows a similar protocol. First, the samples are usually fixed with paraformaldehyde 4% (this step can be skipped if the intent is to track a compound in live cells), following blocking of unspecific sites by incubation with either Bovine serum albumin 3% or goat serum 5%. Next, samples can be incubated with primary antibodies targeting the protein of interest, following incubation with fluorescently labeled secondary antibodies that bind to the primary antibody. For visualization of LDs within cells, LipidTox staining was employed. This technique utilizes a fluorescent probe with high affinity for neutral lipids, enabling their detection and quantification of LDs within the sample. Then, cellular nuclei are counter-stained with Hoechst, and samples are kept in a buffer solution, usually phosphate-buffered saline, under refrigeration until image acquisition. For efficient staining with minimal background signal, it is imperative to include washing steps between each incubation.

Once in the microscope, the light emitted after fluorophore excitation is detectable through specific filters, and the signal is captured by a camera. The image resolution is highly dependent on the microscopy modality, as high and super-resolution instruments employ different light-focusing methods to ensure minimal light dispersion at

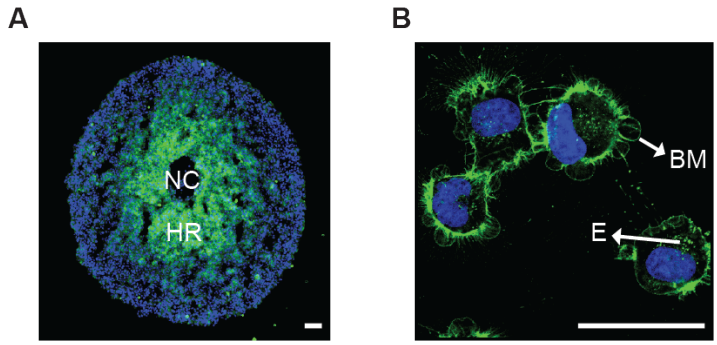


**Figure 11. Fluorescence Microscopy.**
**A**) Example of widefield microscopy utilized to capture CA9 antibody binding in spheroid model of GBM. The pattern of higher expression towards the center indicates the presence of a hypoxic region (HR) around the necrotic core (NC) of the spheroid. **B**) Example of super-resolution image of individual GBM cells stained with anti-CD9 antibody and acquired by AiryScan-supported confocal microscopy allows for detailed visualization of cellular features such as budding membrane (BM) and endocytosed antibody-linked protein (E). In both images, cell nuclei were stained with Hoechst dye and are represented in blue. Scale bars = 50 µm. Source: The author.

the nanometer scale. For observation of general tissue or spheroid protein expression patterns, widefield fluorescence microscopy was utilized in **Papers III** and **IV** by scanning the sample sections in the Zeiss AxioScan Z1 slide scanner (Figure 11A). For high-resolution images at the cellular level, a Zeiss LSM710 confocal fluorescence microscope was utilized throughout **Papers I-IV** for visualization of individual cells at a greater magnification (Figure 11B). Differently than widefield, in confocal microscopy, the light emitted passes through a pinhole aperture, which only enables focused light to reach the detector, reducing diffuse noise signal, and resulting in higher image resolution. Lastly, for super-resolution images, an AiryScan unit can be coupled to the confocal microscope, which further improves light detection thanks to its 32-element array detector, being the chosen modality applied in **Papers II** and **IV**. Each element acts as a pinhole to decrease signal-to-noise ratio and improve resolution for detailed images of subcellular locations for visualization of membrane staining, vesicles, lipids, and other structures down to 10 nm in size.

### *Flow cytometry*

Flow cytometry is a laser-based technology that enables the rapid analysis of individual cells or particles suspended in a fluidic stream and it was used in **Papers II-IV** for the assessment of biotin labeling of surface proteins, endocytosis of biotin-labeled proteins, measurement of phagocytosis of opsonized particles, and uptake assays. In this approach, as each cell transits the flow chamber, it encounters lasers that excite

fluorescently labeled antibodies or particles, emitting light that reveals their presence and abundance in the cell as expressed by mean fluorescence intensity. By capturing and analyzing the fluorescence, flow cytometry generates detailed profiles of individual cells, allowing the identification of diverse cell types, quantification of protein abundance, and monitoring of cellular processes. For flow cytometry quantification, a FACSCalibur (BD Biosciences) or Accuri C6 flow cytometer equipped with a 488 nm wavelength excitation laser and FL1-H detector (533/30 nm) were utilized.

## Cytotoxicity assay

As previously discussed in the **Future treatment possibilities** section, ADCs combine the specificity of antibodies with the potent cytotoxicity of chemotherapeutic payloads, comprising a promising class of targeted therapeutics. Thus, in **Papers II** and **III**, ADC was implemented to display how cellular spacial distribution and hypoxia, respectively, affect the efficiency of ADC-induced cell death when targeting specific proteins. The commercial ADCs used for immunotherapy usually comprise a primary antibody directly conjugated to the cytotoxic payload. However, this direct conjugation is a laborious and costly process that would need to be repeated for each different antibody tested in our studies. Thus, we opted for the use of a commercially available secondary antibody already pre-conjugated to a Monomethyl Auristatin F (MMAF) payload, which, after checking its efficiency, could be complexed with different primary antibodies to test whether their target protein has potential as immunotherapy antigens. In this setting, secondary ADC is first precomplexed to the desired primary antibody binding to a surface protein of interest. Once the primary antibody is bound to its specific target, it piggybacks the secondary-ADC into the cell upon protein internalization, releasing the toxic payload when in the endosome/lysosome, and inducing apoptosis. In the case of MMAF, the payload blocks the polymerization of tubulin, inhibiting cell division, and leading to cell death (Figure 12A).

For cytotoxicity experiments, GBM cells were first seeded in 96-well plates, incubated until 2D and 3D cultures were ready for use (**Paper II**) or pre-conditioned to normoxia/hypoxia 24 h (**Paper III**) before the start of the assay. Next, treatment was initiated by the addition of the respective primary antibodies (anti-EGFR in **Paper II**, and either anti-CXADR, anti-CD81, anti-CD44, anti-CA9, or anti-FXYD6 in **Paper III**) previously pre-complexed with anti-rabbit or anti-mouse secondary IgG monomethyl auristatin F ADC in cell medium for 15 min at 37 °C. Since the experiment readout was based on live imaging with the Incucyte S3 system, 250 nM of Incucyte Cytotox Red Dye was added at the same time as antibody addition to follow cell death. The cytotox reagent is a nucleic acid dye that penetrates the cells, binding to DNA when membrane integrity diminishes as a result of cell death, generating a fluorescent signal (Figure

12B-C). For control conditions, no treatment, primary or secondary antibodies alone, or untargeted primary antibodies (isotype control IgG) with equivalent to the highest ADC concentration were used.

The evaluation of ADC molecule efficacy against TAAs requires a multifaceted assay approach. Several assays were developed for evaluation of proliferation and cytotoxicity, such as MTT and LDH assays, but most techniques are limited to a few readout time points. In this sense, the employment of the IncuCyte live-cell system, *i.e.* an automated system that combines fluorescence microscopy and capture of brightfield images, in these studies enabled frequent monitoring and quantification of cell death (Figure 12D-E) directly within the controlled environment of a standard incubator, eliminating the need for disruptive endpoint analyses and facilitating the understanding of the cellular dynamics over extended periods, ranging from hours to weeks.

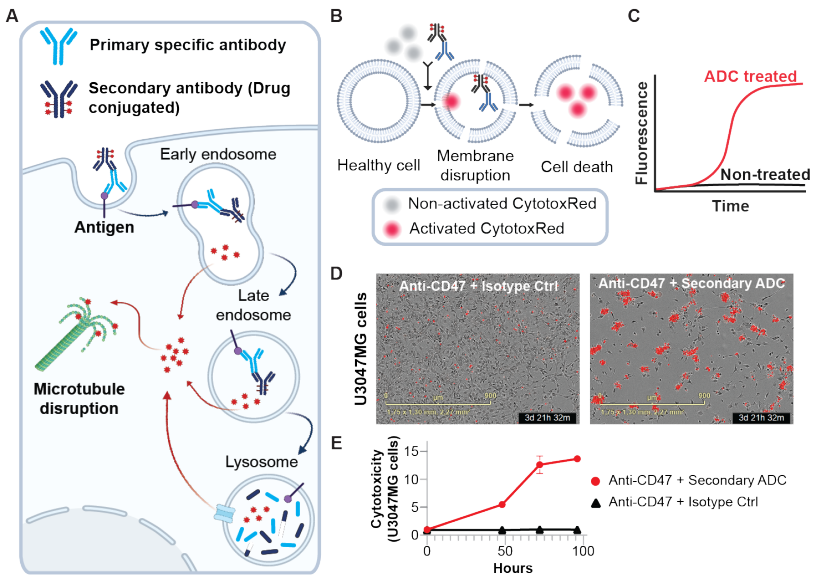


**Figure 12. Cytotoxicity assay.**
**A**) Schematics of the ADC approach utilized. **B**) Schematics representing the Incucyte readout: upon incubation wit ADC + Cytotox reagent, cells that are affected by the toxic payload increasingly lose membrane integrity, allowing CytotoxRed reagent to enter the cell, bind DNA, and generate a red fluorescent signal. **C**) The signal intensity is imaged and analyzed by the Incucyte system, generating comparable and dose-dependent curves. **D**) Incucyte exported images display two groups of U3047MG cells after 3 days of CD47-targeted assay initiation: controls without ADC and ADC-treated cells. **E**) Measurement of CytotoxRed intensity for groups shown in **D**, normalized to cell confluency and time point 0. Made with BioRender.

# The present investigation

This Ph.D. thesis aims to better comprehend the underlying mechanisms sustaining the adaptation of GBM cells to the stressing factors within the TME. It focuses on understanding how hypoxia modulates GBM regarding the landscape of targetable surface proteins and its effects on scavenging cells. Additionally, this thesis presents a potential mapping technique applicable for the profiling of tumor cell surface and explores the pro-malignant dynamics between TME lipids and stressed glioma cells. Finally, with a clearer therapeutic aim, we set out to develop a methodology for surface target identification using *in vitro* models and intact mouse and patient brain tumor specimens.

## Specific aims of the Thesis

- To investigate how lipid loading impacts the cross-talk between hypoxic cells in different GBM models. (Paper I)
- To develop a method to efficiently retrieve and identify surface-bound internalizing proteins in freshly resected tumor samples. (Paper II)
- To uncover potential targetable proteins representative of GBM cell adaptation to hypoxic stress. (Paper III)
- To characterize tumor-associated foamy macrophages present in the hypoxic GBM TME. (Paper IV)

**Author Contributions**

The following describes the thesis author's contributions to the included papers:

**Paper I** - Bioinformatics analysis (*e.g.* Differential gene expression analysis of Hypoxia/lipid loading data and Pathway analysis); Graphical summarization and; Manuscript writing and reviewing.

**Paper II** - Patient sample biotinylation; SURFME filter generation; Bioinformatics analysis (*e.g.* Gene set enrichment analysis and Differential protein abundance analysis); Graphical summarization; Manuscript writing and reviewing.

**Paper III** - Sample preparation for transcriptomics/proteomics; Bioinformatics analysis (*e.g.* Differential gene/protein expression analysis and ranks generation); Experimental validation (Stainings, Flow cytometry, Western blot); Graphical summarization; Manuscript writing and reviewing.

**Paper IV** - Bioinformatics analysis (*e.g.* TAF signature analysis and Public database evaluation); Graphical summarization; Manuscript writing and reviewing.

# Main results and conclusions

The rationale behind **Papers I-IV** and their main results are summarized here. For detailed reading, please consult **Papers I-IV** in the appendix section of this thesis.

## Paper I

*Extracellular lipid loading augments hypoxic paracrine signaling and promotes glioma angiogenesis and macrophage infiltration*.

Studies by our group and others have shown that hypoxic conditioning leads tumor cells to increase the scavenging and storage of lipids for adaptive advantages (*e.g.* oxidative stress management) and that hypoxic GBM regions often associate with lipid-dense areas under magnetic resonance spectroscopy. In light of this, we evaluate in **Paper I** whether the link between lipid species and low oxygen tension goes beyond autocrine metabolic benefit, exerting other paracrine roles in the TME to confer tumoral advantages during hypoxic adaptation. Thus, we start **Paper I** by experimentally replicating the lipid-loaded phenotype in hypoxic U87MG GBM cells supplemented with an external lipid source (Figure 13A) and validating its presence within the hypoxic areas of GBM patient tumor cells. Upon transcriptomic profiling of *in vitro* and patient samples, we found that LD loading potentiates the hypoxic response, and 430 genes were found to be commonly upregulated by this LD phenotype (Figure 13B), converging in pathways such as angiogenesis, stromal remodeling, macrophage recruitment, LD storage, etc.

Immunoassay interrogation of the secreted proteome of U87MG cells revealed that several proteins were upregulated by hypoxia and further enhanced by lipid loading, including VEGFA and HGF, which were also secreted in the plasma of GBM patients (pre-surgery) and associated with worse prognosis. Even though LD-loading potentiated hypoxic response at the protein level (in comparison to hypoxia versus normoxia alone), this effect was independent of the canonical signaling as HIF-1$\alpha$ protein abundance remained unaltered by LDL supplementation (Figure 13C). Instead, the transient increase in the phosphorylation of cyclic AMP-response-element binding protein

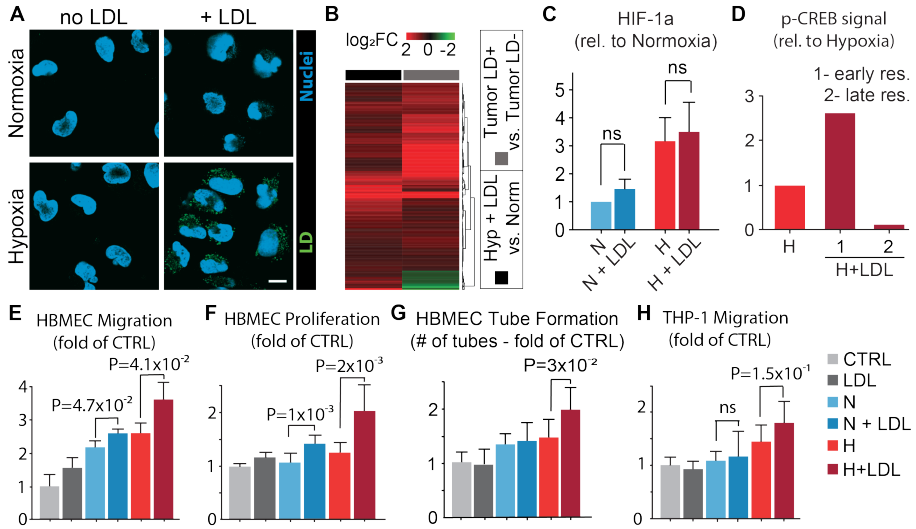


**Figure 13. Effects of LD-phenotype in 2D models.**
**A)** IF images displaying the presence/absence of LDs in Normoxic/Hypoxic GBM cells (U87MG) supplemented or not (+/-) with extracellular lipids (LDL, 50 ug-ml). Scale bar, 20 µm. **B)** Heatmap of upregulated genes in Hypoxic + LDL vs Normoxic GBM cells and LD+ vs LD- areas from GBM patient tumors. **C)** Western blot quantification of HIF-1α expression (U87MG lysates). **D)** Phosphokinase antibody array analysis of lysates from hypoxic U87MG cells. early res = after short (5-30 min) LDL incubation. late res = after long (45-90 min) LDL incubation. **E)** Quantification of HBMEC transwell migration towards different CMs (serum-free medium (grey), full medium from normoxic cells (blue), or full medium from hypoxic cells (red) +/- LDL) after 6 h incubation. **F)** Quantification of HBMECs proliferation after 72 h of incubation under the same conditions as described in E. **G)** Quantification of Matrigel tube formation after 20 h of cell incubation at the same conditions as described in E. **H)** Human-derived monocytes differentiated into macrophages (THP-1) transwell migration quantification towards different CMs (same as described in E) after 6 h incubation. Modified with permission from Offer *et al.*, 2019 [264].

(CREB) (Figure 13D), which is known to cooperate with HIF-1α to induce VEGFA and other hypoxia-induced proteins, is proposed as a possible mechanism for such boost.

Upon assessment of how the secretome of LD-loaded cells affects EC (HBMEC) behavior, we found that they display increased migration towards the conditioned medium of LD-loaded hypoxic glioma cells (Figure 13E), as well as higher proliferation (Figure 13F) and angiogenic (tube formation) potential (Figure 13G). When evaluating the behavior of macrophages (THP-1), hypoxic LD+ conditioned medium was again a potent inducer of cell migration (Figure 13H), showing that the LD phenotype can impact the functional stromal crosstalk of important players of the TME. We could also confirm the observed stromal impact of LD+ tumor cells in different *in vivo* models. Mice tumors grown from the injection of LD+ GL261 tumor cells (Figure 14A and D) presented a noticeable rise in the infiltration of TAMs (Figure 14B and E, black bars)

and angiogenesis (Figure 14C and F, black bars), both of which could be heightened due to systemic hyperlipidemia alone (Figure 14E and F, dark grey bars).

These findings show the role of lipids in the manipulation of adaptive responses of GBM cells to hypoxia, highlighting their strong contribution to paracrine signaling, angiogenesis, and immune cell recruitment, and displaying lipids as interesting potential targets for the development of disruptive therapies to improve treatment outcomes for patients with GBM. The recruitment of scavenging cells such as TAMs is of special interest, warranting further studies for the mechanistic understanding of this phenomenon.

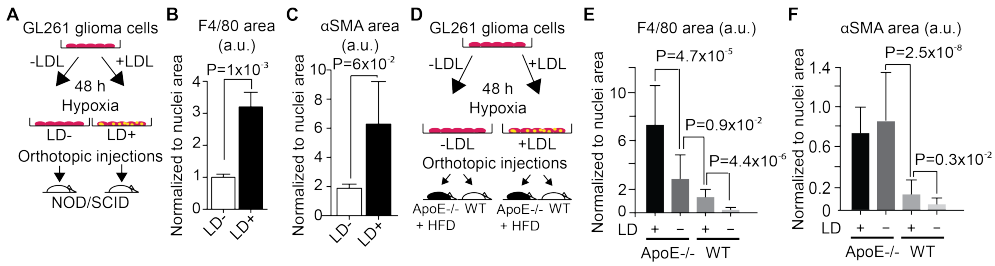


**Figure 14. Effects of LD-phenotype in mouse models.**
**A)** GL261 cells, primed or not with extracellular lipids, were incubated in hypoxia for 48 h and then injected (50K cells) into NOD/SCID mice brains. **B)** Macrophage quantification (as measured by the F4/80 marker) in frozen LD+ or LD- mice tumor sections, normalized to cell nuclei (Hoechst). **C)** Quantification of αSMA-positive pericyte-like vascular cells in frozen LD+ or LD- mice tumor sections, normalized to cell nuclei (Hoechst). **D)** GL261 cells were treated as described in **A**, and injected (100K cells) into the brains of wildtype mice on a chow diet or of ApoE knockout mice under a high-fat diet (HFD). **E and F)** Quantification of macrophage infiltration and angiogenic response as shown in **B and C**, respectively, from frozen tumor sections collected from the experiment shown in **D**. Modified with permission from Offer *et al.*, 2019 [264].

## Paper II

*Landscape of surfaceome and endocytome in human glioma is divergent and depends on cellular spatial organization.*

Finding relevant targets for the development of improved antitumoral strategies is a concerning bottleneck in research. To aid this step, in **Paper II**, we sought to demonstrate the applicability of the Tumor Surfaceome-Mapping (TS-MAP) approach, a protocol developed by the authors to efficiently label surface proteins (*i.e.* the surfaceome) of cells from different origins with the use of a reversible membrane-impermeable biotin tag (Figure 15A). While allowing for the labeling of the surfaceome, the TS-MAP

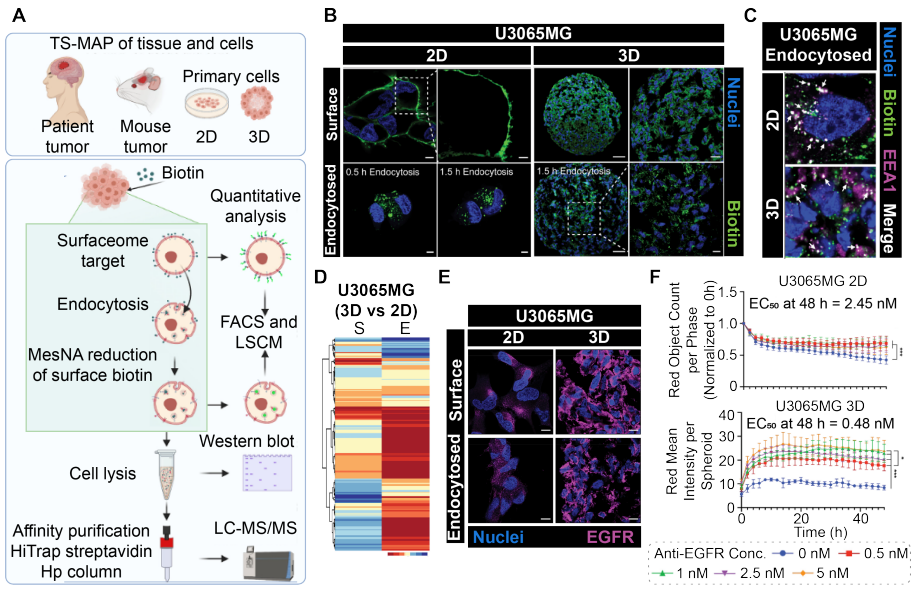


**Figure 15. The TS-MAP protocol.**
**A)** Schematic outlining the accepted sample types and procedures for uncovering the surfaceome and endocytome. **B)** IF of surface and endocytosed biotinylated proteins in 2D and 3D GBM models. Scale bars, 5 µm [2D], 2 µm [2D Inset], 50 µm [3D, left], and 20 µm [3D, detail]. **C)** Co-localization of endocytosed biotin and anti-EEA1 signals. Scale bars, 2 µm. **D)** Heatmap of diverging protein abundances in 3D vs 2D. **E)** IF of anti-EGFR signal in 2D and 3D GBM models. Scale bars, 10 µm. **F)** Cytotox signal after treatments with increasing concentrations of anti-EGFR ADC, read at every 2 h in 2D (upper graph) and 3D (lower graph) GBM models. Modified with permission from Governa *et al.*, 2022 [244].

also allows for the identification of internalizing surface proteins (*i.e.* the endocytome) in the same workflow, thus facilitating the identification of potential candidate targets for the design of immunotherapies such as ADC. To validate the TS-MAP efficiency, we first applied it to 2D and 3D models of the primary GBM culture U3065MG and

showed that biotin tagging can efficiently label surface proteins of both models (Figure 15B, top row). Moreover, when under endocytosis-permissive conditions, the labeled proteins were seen to be efficiently internalized, which could be visualized upon reduction of the remaining surface signal (Figure 15B, lower row) and proven to result from active membrane-budding endocytosis via co-localization of the biotin signal with the early endosome antigen 1 (EEA1) (Figure 15C) and with endolysosomal vesicles.

One of the major endpoints of the TS-MAP is the proteomic profiling of labeled proteins. Thus, purified surfaceome and endocytome samples of biotinylated 2D and 3D U3065MG cells were profiled by label-free quantitation LC-MS/MS, following proteomic data filtering with our in-house curated dataset of *bonafide* surface proteins (SURFME), displaying as a result that 3D cell culture can highly impact the surfaceome pattern of cells, especially regarding their endocytic potential (Figure 15D). Among the TS-MAP hits of proteins with increased surface expression and higher endocytosis in our 3D model was EGFR (Figure 15E), the latter of which translated into increased ADC effect (Figure 15F), supporting the ability of the protocol in capturing biologically relevant proteins.

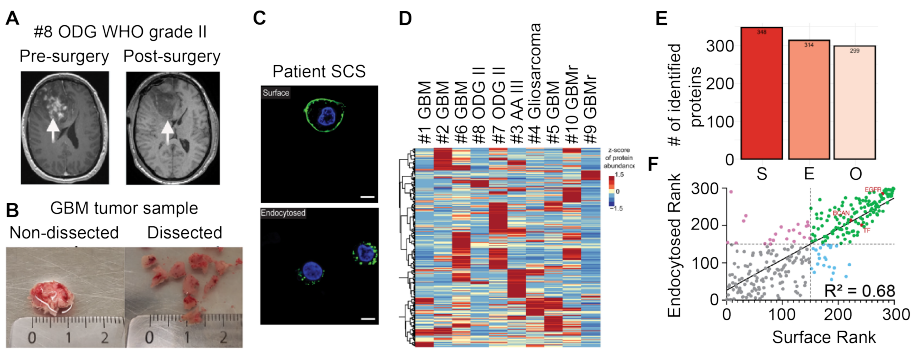


**Figure 16. TS-MAP application to a patient glioma cohort.**
**A)** Patient MRI presurgery and postsurgery within 48 h. **B)** Example of freshly resected tumor dissected into 0.3-0.5 cm pieces prior to biotinylation. Ruler scale in cm. **C)** IF imaging of single-cell culture showing specific plasma membrane labeling (top) and endocytosed biotin signal in patient sample (bottom). Scale bars, 5 µm. **D)** Heatmap of SURFME protein abundances among different patient tumor diagnoses. **E)** Number of SURFME proteins identified in the same tumor tissue in surface, endocytosed fractions, and their overlap. **F)** Abundance-based ranking of proteins identified in the same sample shown in **E** (lowest abundance = 1, highest abundance = 299), and divided into four categories. Proteins in the top right quadrant present with high surface abundance and efficient endocytic capacity. Modified with permission from Governa *et al.*, 2022 [244].

Lastly, we sought to apply the TS-MAP protocol to a pilot cohort of 10 glioma patients for profiling of intact tumor samples (examples of collected tumor tissues in Figure 16A and B), where we showed that the comprehensive biotinylation performed

to intact samples is stable to single-cell suspension dissociation (Figure 16C) and that proteomic profiling reproduces the tumor heterogeneity commonly seen in the clinical setting (Figure 16D). Furthermore, we show that, in cases where tumor volume allows, the protocol can be utilized to characterize both surfaceome and endocytome proteins in the same sample with a good overlap between the number of proteins identified in both fractions (Figure 16E), allowing for the ranking of abundant proteins in a patient-specific manner (Figure 16F). Overall, our results suggest that the TS-MAP could be employed for the personalized identification of targets for the development of tailored therapy. Moreover, our protocol can be a powerful tool for the identification of proteins of interest in a myriad of samples such as mouse tumors and cell lines.

## Paper III

*Decoding of the surfaceome and endocytome identifies potential cell-surface target antigens in the hypoxic niche of glioblastoma.*

**Paper III** dives into the hypoxic response of GBM, bringing up possible targets that could help direct future therapeutical developments against stress-adapted cells and displaying the use of the TS-MAP protocol for uncovering targets induced by TME events (Figure 17A).

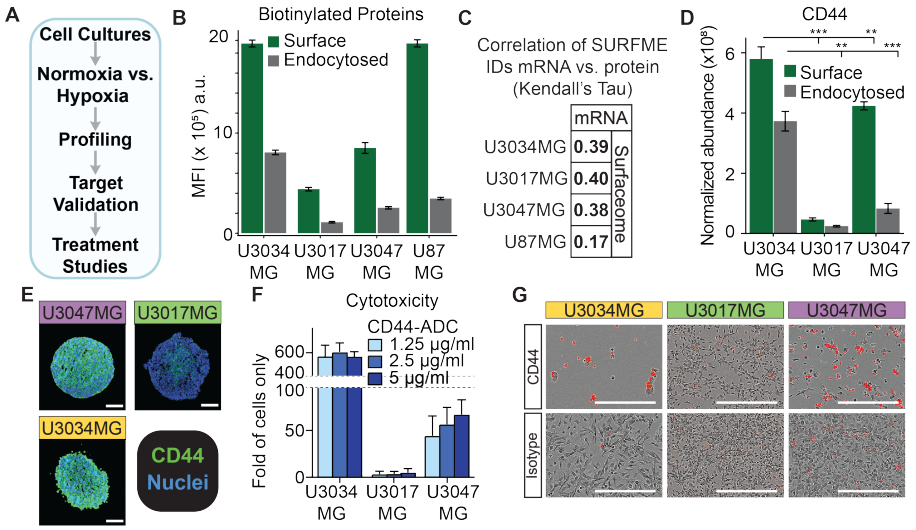


**Figure 17. TS-MAP application to GBM cell cultures.**
**A)** Schematics of the study steps. **B)** FACS quantification of biotinylated surfaceome and endocytome in GBM cell cultures. **C)** Correlation of SURFME identity between transcriptomics and TS-MAP proteomics. **D)** Comparative surface and endocytosed CD44 protein abundance between GBM cultures. **E)** IF staining for CD44 in GBM spheroids. Scale bars, 200 µm. **F-G)** Quantification and representative images of concentration-dependent cytotoxicity over time analyzed by live-cell imaging of GBM cells treated with anti-CD44 ADC. Scale bars, 450 µm. Modified with permission from de Oliveira *et al.*, 2024 [265].

We started the study by applying the TS-MAP protocol to primary GBM cells representative of different transcriptional subtypes and the U87MG cell line. We observed that the global amount of surface and endocytosed proteins drastically changed between cell types (Figure 17B), usually correlating well with average cell size. Upon proteomic and transcriptomic profiling, we found that proteomics results did not correlate with transcript abundance of corresponding surface-protein coding genes (Figure 17C), evidencing that transcriptomics, while useful for uncovering mechanisms of action, is not the optimal layer of data for the selection of possible therapeutic targets and

reinforcing the importance of protein-based studies for this type of research. Then, to challenge the accuracy of the proteomics results we chose the well-known and abundantly GBM-expressed protein CD44 as a proof-of-concept target, which presented clear distinct protein abundance levels among the different primary GBM cells, both at the surface and endocytosis, which could be promptly validated experimentally (Figure 17D). Indeed, surface antibody binding targeted against CD44 (Figure 17E) and its internalization followed the same pattern as seen in the TS-MAP quantitation, where it displayed very-low abundance in U3017MG cells, high abundance in U3034MG cells and intermediary levels in U3047MG cells. Moreover, the differences in internalization translated into dramatic variations in the sensitivity to anti-CD44 ADC treatment (Figure 17F-G).

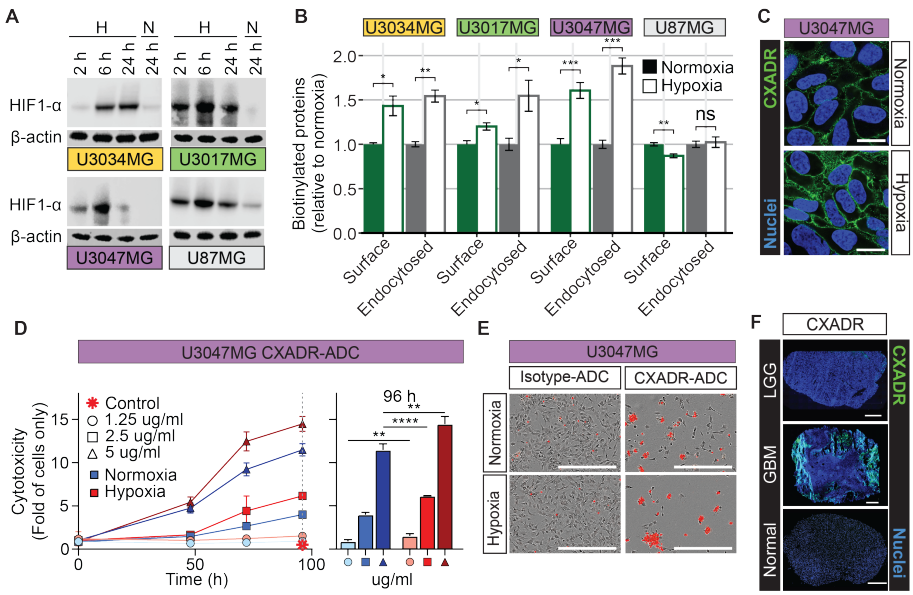


**Figure 18. Identification of hypoxia-induced proteins.**
**A)** HIF1-α immunoblotting of cell lysates from normoxic and hypoxic cells at the indicated time points, with β-actin as loading control. **B)** FACS quantification of biotinylated surface and endocytosed proteins in normoxic and hypoxic GBM cell cultures, normalized for normoxia. **C)** CXADR protein expression in hypoxic/normoxic U3047MG 2D cultures. Scale bars, 20 µm. **D)** Left: Fold CytotoxRed signal over time by different concentrations of anti-CXADR ADC, normalized to the cytotoxicity of cells only and phase confluency. Right: Quantification of cytotoxicity at 96 h of treatment. **E)** Representative images from data shown in **D**. Scale bars, 450 µm. **F)** IF validation of CXADR in LGG, GBM and Normal brain tissue. Scale bars, 1000 µm. Modified with permission from de Oliveira *et al.*, 2024 [265].

Once tested with normoxic cells, we then applied the protocol to cells conditioned to hypoxia (1% $O_2$, 24 h) and presenting HIF1-α activation (Figure 18A), and we found that hypoxic conditioning increases the global surfaceome and endocytome abundance

in primary GBM cells when compared to normoxic cells (Figure 18B) and lead to the upregulation of several hallmark targets of hypoxic response such as GLUT3 and CA9 proteins.

Additionally, protein upregulation in response to hypoxia was seen to be highly divergent. Shared inductions were scarce and poorly correlated with upregulations observed at the transcriptional level. Next, we proceeded to identify and validate possible hypoxia-induced targets on a cell-type basis. Interestingly, we found the CXADR protein as a strongly hypoxia-induced target in U3047MG cells (Figure 18C), which translated into an efficient dose and time-dependent cytotoxic effect upon anti-CXADR ADC treatment significantly greater in cells conditioned to hypoxia (Figure 18D-E). Moreover, staining with anti-CXADR antibody in human tissues showed high CXADR protein abundance in GBM while presenting very low or absent presence in low-grade tumor and normal brain tissue (Figure 18F). In addition, 4 other potential targets induced by hypoxia were identified such as CD47 in U3047MG cells, CD81 as a shared protein overexpressed in U3047MG and U3017MG cultures, FXYD6 in U3017MG, and BSG as a shared target in U3017MG and U3034MG cells. In summary, **Paper III** exploits the protein remodeling of GBM cells when under hypoxic stress, underscoring the value of proteomics in dissecting the complexity of the surface-endocytome and identifying potential therapeutic targets.

## Paper IV

*Lipid droplet-loaded macrophages as a targetable protumorigenic immune cell entity in human glioblastoma.*

TAMs are the main type of immune cells present in GBM (approx. 50% of the tissue stromal cells), being usually rewired to an immunosuppressive state and associated with increased tumor aggressiveness. As observed in **Paper I**, the GBM TME displays abundant lipid content, which modulates the stromal cell crosstalk for the attraction of macrophages within the hypoxic (and immunosuppressive) niche. Moreover, in several pathologies (*e.g.* atherosclerosis, infectious and neurodegenerative disease), the combination of scavenging cells and a lipid rich environment has been seen to result in LD accumulation within this type of stromal cell, which acquires a foamy phenotype. Therefore, in **Paper IV** we identify the presence of tumor-associated foam cells (TAFs) as a previously unidentified immune cell population within the GBM TME, exhibiting distinct pro-tumorigenic characteristics.

We initially found that the lipid-loaded phenotype is a characteristic phenomenon of GBM tumors, absent or scarcely present in the normal brain or low-grade tumor tissues (Figure 19A-B). Moreover, we show that GBM macrophages ($CD68^+$), in particular, display a higher LD-phenotype than macrophages from low-grade tumors (Figure 19C). To better understand these cellular entities, $CD68^+/LD^+$ (TAF) and $CD68^+/LD^-$ (no TAF) areas were microdissected from brain tumor tissues (Figure 19D) and transcriptionally profiled, revealing that more than 2000 genes were impacted by lipid accumulation during TAMs transformation into TAFs (Figure 19E). Among the enriched pathways, TAFs were seen to upregulate genes associated with hypoxia and response to lipids (Figure 19F), and TAF signature score (54 genes) was seen to strongly associate with pseudopalisading (PP) regions more than any other GBM compartment (Figure 19G). Furthermore, upon dichotomization of patient cohorts according to TAF presence, we found that increased amounts of $CD45^+$ and LD+ cells ($TAF^{high}$) conferred a 4-times higher risk of death (Figure 19H) independently from tumor size or necrosis volume, suggesting that the lipid rewiring previously known to impact adaptation of cancer cells can also impact the dominating TAM population, potentially causing broader and patient-reaching consequences.

Next, to comprehend the mechanism by which TAMs become TAFs, we explored the contribution of EVs, an abundant lipid source within the GBM TME, to the foamy phenotype by differentiating primary human monocytes ($CD14^+$) from peripheral blood into macrophages and incubating them with isolated EVs or conditioned medium from patient-derived GBM cells (Figure 20A). After incubation, in both cases, we observed an induction of lipid formation, that was greatly pronounced in hypoxic conditions and

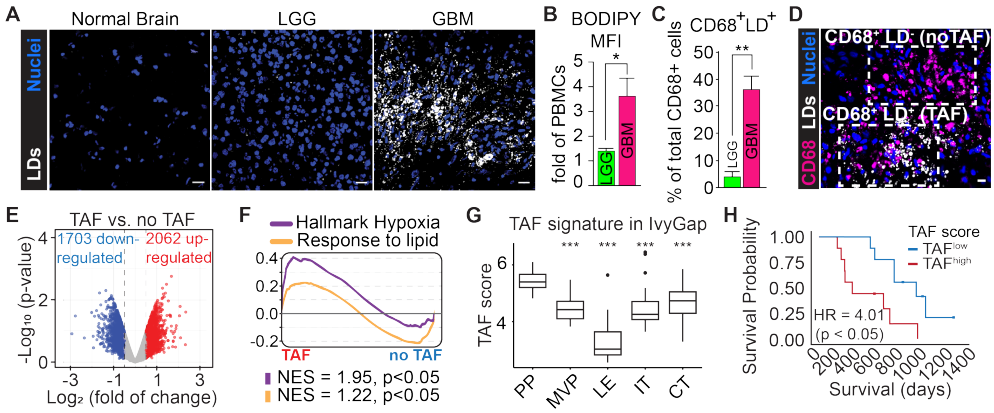


**Figure 19. Unveiling TAFs within the GBM TME.**
**A)** IF LipiTOX staining of LDs in different human tissues. Scale bars, 20 µm. **B)** FACS quantification of neutral lipids staining with BODIPY in 4 patient-derived GBM cultures. **C)** Quantification of the fraction of $CD68^+LD^+$ of total $CD68^+$ cells in GBM and LGG (n = 6 patients, 53 areas/group). **D)** Example of $CD68^+LD^-$ (no TAF) and $CD68^+LD^+$ (TAF) areas (dashed white squares) captured by LCM from 8 patients. **E)** Gene expression in TAF vs. no TAF. **F)** GSEA results showing enrichment of "Response to Lipid" and "Hallmark Hypoxia" pathways in TAF samples vs. no TAF. **G)** TAF score strength in different GBM niches. PP = Pseudopalisading; MVP = Microvascular Proliferation; LE = Leading Edge; IT = Infiltrating Tumor; CT = Cellular Tumor. **H)** Kaplan-Meier plot of survival against TAF score dichotomized at the median. Modified with permission from Governa *et al.*, 2024 (Manuscript unpublished).

efficiently abrogated when cells received LD-inhibiting drugs (*e.g.* DGAT1i) (Figure 20B), showing the foamy formation to be dependent on the enzymatic triglyceride processing. Interestingly, we found a significant reduction of the phagocytosis potential in TAF vs. no TAF (Figure 20C) combined with an increased angiogenic drive (displayed by the increased migration of ECs shown in Figure 20D), for example), both of which could be partially restored upon treatment with LD-inhibiting drugs. Transcriptomic profiling of ECs treated with conditioned media of macrophages treated or not with EV alone or EV + DGAT1i showed that several genes responded to the counteraction of pro-angiogenic activation (Figure 20E).

Lastly, we evaluated how these findings would translate in an *in vivo* setting by co-injecting U3054MG GBM cells mixed with macrophages (TAF or noTAF) alone or GBM cells mixed with foamy macrophages treated with Tracsin C, a drug that inhibits lipid-chain elongation (Figure 20F). We found that the presence of LD-loaded macrophages significantly accelerated tumor formation and this effect could be promptly reversed by treatment with Triacsin C (Figure 20G), which also reversed angiogenic drive and overall tumor LD-loading. Taken all together, **Paper IV** demonstrates that foamy macrophages contribute to the aggressive phenotype of GBM tumors, exhibiting a pro-tumorigenic behavior, increased angiogenesis, impaired phagocytosis, and worse

patient outcome. Importantly, we demonstrate that targeting key enzymes involved in LD formation effectively disrupts TAF functionality, establishing TAFs as a prominent immune cell entity of GBM and a potential target for future therapeutic development. These results are, as per the date of this thesis, unpublished and under consideration for publication.

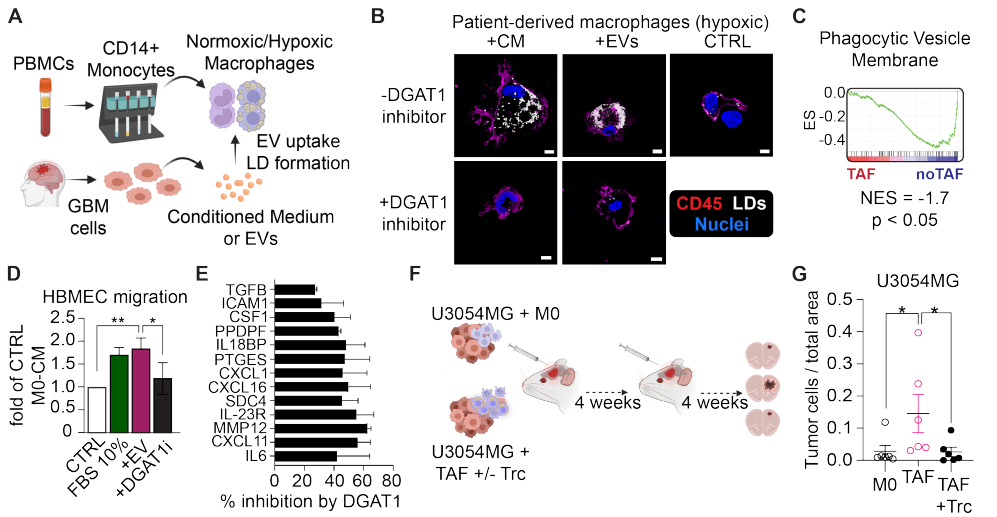


**Figure 20. TAM LD acquisition and reversion.**
**A)** Schematics of *in vitro* experiments with primary macrophages and GBM cells. **B)** IF of $CD45^{+}$ cells from GBM after 48 h hypoxia in fresh medium (CTRL), conditioned medium (CM), or EV, with or without DGAT1 inhibitor (DGAT1i; 5 µM). Scale bars, 5 µm. **C)** GSEA showing depletion of phagocytosis-related pathways in TAF vs. no TAF hypoxic Macrophages. **D)** HBMEC transwell migration assay over 6 h towards TAM conditioned medium (CTRL), fetal bovine serum 10% (FBS 10%), conditioned medium from TAF cells treated with EVs (+EV) or conditioned medium from TAF cells treated with EVs and DGAT1 inhibitor (+DGAT1i). **E)** % of DGAT1i-dependent inhibition of HBMEC gene expression when comparing ECs incubated (6 h) with conditioned medium from TAMs or TAFS, with or without DGAT1i. **F)** Schematics of *in vivo* experiments for the co-injection of U3054MG GBM cells + TAMs, TAFs, or TAFs + Triacsin C in NOD SCID mice. After 4 weeks, tumors were re-injected with macrophages and mice were sacrificed after another 4 weeks. **G)** Mice tumor area as measured by quantification of human cell nucleus antigen (HuNu) (n = 6 mice/group). Modified with permission from Governa *et al.*, 2024 (Manuscript unpublished).

# General discussion and future directions

In this thesis, we sought to elucidate some of the adaptive events of tumor cells in hypoxic environments. We explored the crucial role of lipids within this niche and introduced a new tool for detailed profiling of the cell surface proteome (surfaceome). We emphasized that hypoxic adaptation is not a singular event, but rather a complex interplay involving refined cellular processes, multilateral interactions between tumor components, and dynamic remodeling of the tumor cell surface. These adaptations were observed to have a significant impact on tumor aggressiveness, influencing the behavior of immune effectors and ECs, and ultimately contributing to enhanced tumor growth and invasive potential.

In **Paper I**, we studied the contribution of lipids to hypoxia-driven tumor development. We observed that hypoxic GBM cells in the presence of extracellular lipids acquired a lipid-loaded phenotype, a phenomenon also observed in the hypoxic tumor niche of GBM patient tumors. Such phenotype further induced and maintained the expression of hypoxia-regulated genes involved in TAM recruitment, angiogenesis, and matrix remodeling, even after reoxygenation. Furthermore, combined tumor cell lipid loading and systemic hyperlipidemia significantly increased TAM infiltration and decreased survival of *in vivo* models. These findings suggest that, besides its bioenergetic role, LDs also play an important paracrine role in shaping the TME towards tumorigenesis, and that this process happens partly through macrophage recruitment. An increasing number of studies have taken on the task of elucidating the mechanisms of hypoxia-driven crosstalk between tumor cells and TAMs (reviewed at [89]), revealing exosomal micro-RNAs and interleukins as the main mediators of this process. Interestingly, the tumor-derived exosomal miR-301a-3p was seen to be upregulated by HIFs (1 and 2) and, upon transferring into TAMs, it promoted matrix remodeling and invasion [89]. However, the contribution of lipids to this mediation is yet to be fully understood.

Besides lipids, cell surface modification was also observed to be a significant consequence of adaptation to hypoxia. Previous profiling of the surfaceome of $CD8^+$ T cells submitted to hypoxia revealed a large-scale surfaceome alteration consistent with metabolic reprogramming and leading to immunosuppression [266]. Therefore, in our next studies, we sought to explore hypoxia-induced surfaceome alterations of GBM cells, as these modifications could further orchestrate interactions with the microenvironment, influencing tumor aggressiveness. To efficiently do so, the utilization of advanced techniques to map the surface proteins was paramount. Thus, in **Paper II** we

presented the TS-MAP method as a new tool for the comprehensive identification of surface antigens. In contrast to commercially available biotinylation-based methods, the TS-MAP demonstrates a key strength: its ability to capture endocytosed surface proteins (endocytome) in a high-throughput manner. This unique feature provides a crucial layer of information for deciphering the patterns of uptaken proteins by tumor cells. To the author's knowledge, **Paper II** was the first PubMed-indexed study to present the term endocytome, referring to the large-scale proteomic identification of endocytosed proteins. Some of the previous techniques used for the characterization of this fraction of proteins heavily relied either on (1) the isolation and purification of endosomes/lysosomes profiled by LC-MS/MS [267], (2) on the selection of few targets for antibody-based colocalization with lysosomal markers (*e.g.* LAMP1) [268], or (3) fluorescence emission by labeled targeted antibody upon endocytosis (*e.g.* pH-sensitive dye pHrodo Red) [269]. Moreover, endosomal-based proteomics does present with a set of limitations ranging from the overrepresentation of classical endosome proteins in the proteomic data (which can mask the identification and proper quantification of the lower abundance cargo proteins), to the experimental difficulty of the isolation/purification methods, and the high risk of sample contamination with non-endocytosed proteins due to incomplete separation of endosomes from other cellular components.

Upon assessing the applicability of the TS-MAP, we established its compatibility with 2D and 3D cell culture models, as well as with freshly resected intact tumor pieces. The profiling of cell cultures allowed us to visualize how spatial organization impacts protein endocytosis patterns, consequently affecting the efficacy of ADC-targeting approaches. Additionally, the profiling of the intact tumor tissues identified patient-specific hotspots of upregulated proteins that represent a valuable resource for the development of personalized therapies, especially ADCs and CAR-T cells. Despite being resourceful, the method presented in **Paper II** is not without its limitations. It remains to be assessed how glycosylation affects biotin binding and the subsequent LC-MS/MS quantitation of such proteins. Moreover, the sample preparation protocol could benefit from further refinements such as the improvement of the sample precipitation protocol for better mass-spec representation of low-abundance proteins. Also, the method could be, in the future, combined with single-cell proteomic methodologies for the assessment of spatially resolved surfaceome/endocytome.

In **Paper III**, we then made use of the TS-MAP protocol to elucidate the dynamic changes occurring on the surface of hypoxic cells. First, we provide a proof-of-concept on normoxic cells to verify that protein quantitation, exemplified by CD44 protein, would reflect the abundance observed in 2D and 3D GBM cell cultures, patient tissue stainings, and would translate into efficient targeted antibody internalization and ADC response. Next, we observed a robust hypoxic response across different primary GBM cultures and U87MG cells, indicating the significant impact of hypoxia on the

remodeling of the surface-endocytome in GBM, which provided a repertoire of potential target antigens expressed by hypoxia-adapted cells. Interestingly, such repertoire was poorly correlated with the integrated transcriptomic profiling data, possibly due to intense modulations of intermediary structures impacting the availability of the actual end protein, such as mRNA degradation and protein post-translational modifications. These phenomena emphasize the importance of proteomics-based explorations for the informed choice of candidate target antigens. Moreover, the landscape of hypoxia-induced proteins exhibited substantial heterogeneity between cells representative of different GBM subtypes, which required the selection of distinct surface targets for subtype-specific ADC challenging. This illustrates the heterogeneity of GBM tumor cells and underscores that, as seen in previous studies, targeting of single antigens is most likely to affect a limited portion of the tumor cells while leaving other subpopulations unaffected. Despite Paul Ehrlich's ideation, ADCs as monotherapeutical strategies are unlikely to act as a magic bullet against all malignant cells of GBM.

From a technical perspective, the 2D cell model utilized in **Paper III** contrasts with the conclusions of **Paper II**, where the utilization of 3D models showed increased ADC efficiency. The use of 3D cell models is undoubtedly suitable for explorations of broadly expressed proteins, such as EGFR, as presented in the study. However, challenging of hypoxic targets with ADC strategies in spheroids proved enigmatic. The effects of the cytotoxRed-based quantification of the cytotoxicity induced by ADCs in the hypoxic core of spheroids were absconded by interference from the increased rate of apoptosis due to hypoxia/necrosis alone. Alternatively, evaluating spheroid shape changes is also a way to assess cell death, but this also proved problematic for assessing hypoxia-restricted apoptosis due to the maintenance of the overall spheroid area despite core cell death. This confounded data analysis, contrasting to the spheroid-wide loss of integrity observed when targeting niche-unspecific proteins. Thus, while useful, the utilization of more complex 3D models proved to be challenging for the assessment of proteins with limited spatial expression. Meanwhile, 2D models allowed for the isolation and homogenization of the hypoxic effects, which were then validated with sections from 3D and patient tumor slides. In future endeavors, the investigation of the TS-MAP identified hypoxic targets in **Paper III** will further evaluate their role in tumorigenesis and prioritize the most promising candidates for clinical development.

Lastly, in **Paper IV**, the role of TME lipids is further explored. In this study, we characterized a set of lipid-loaded TAMs, defined here as tumor-associated foam cells (TAFs), which are formed upon scavenging of EVs released by tumor cells and found enriched in hypoxic/pseudopalisading regions of GBM (but not in LGG). Lipid-loaded macrophages were previously associated with other conditions such as Parkinson's disease and atherosclerosis [270]. However, it had not been clearly described as a GBM-associated entity until now. We speculate that BMDM-derived macrophages are ini-

tially attracted to the tumor's hypoxic niche in attempts to engage in the clearance of toxic lipid species and to initiate anti-tumoral signaling, turning pro-tumorigenic after prolonged exposure to the hostile TME. Tumor cells were seen to secrete IL-1B, which induces the expression of MARCO scavenger receptor by the macrophages, promoting the scavenging of *e.g.* oxidized LDLs [271]. Also, CD36 at the surface of TAMs mediates free fatty acid removal. Over time, the excess of LD buildup in macrophages stimulates signaling via PI3K-gamma, increasing the expression of the immunosuppressive molecule PD-L1, and leading to a reduction of phagocytic activity [271]. In **Paper IV**, we observed that both TAFs and primary macrophages loaded with LDs displayed clear pro-angiogenic signaling, fostering communication with ECs as observed previously in **Paper I**. Thus, both GBM cells and TAMs obtain such phenotype and reinforce angiogenic signaling and immunosuppression. However, the temporal course of these observations is still to be further explored.

Next, we sought to investigate the potential outcomes of TAFs presence in patient-derived data. After stratification of a pilot GBM cohort based on the presence of TAFs in the tumor tissue, enrichment of the TAF score was seen to confer a 4x higher risk of death regardless of tumor size and necrotic volume. Upon TCGA GBM data analysis, the risk of death was nearly 2x increased for patients enriched for TAF-signature genes, and the signature was strongly associated with EMT promotion. Given that our data reiterates the role of tumoral LDs as active signaling molecules of TME rather than passive storage structures, it is expected that other TME features culminate into aberrant lipid signaling and TAF formation. Thus, reprogramming of the LD phenotype can be a promising therapeutical approach to deplete tumor-promoting TAFs while maintaining the population of anti-tumoral macrophages unharmed. Therefore, we explored this strategy in **Paper IV** by making use of triglyceride-disrupting drugs (*e.g.* DGAT1 and Triacsin C) which were able to reverse some of the effects associated with lipid-loaded cells. Administration of DGAT1 inhibiting drug displayed promising results against GBM TAFs, but also against foamy macrophages in a colon cancer preclinical model as seen by Wu *et al.* [272]. The inhibition was seen to redirect macrophage phenotype back to an anti-tumoral state that impaired tumor growth. Moreover, PI3K-gamma inhibition can also be a promising strategy to revert the M2-like polarization of TAMs under lipid accumulation, which also showed beneficial effects in preclinical gastric tumor models. As shown by Conza *et al.* [273], lipid loading can cause endoplasmic reticulum stress, which assists in macrophage polarization. This was reversible by inhibition with LXR agonist, which ceased lipid-induced immunosuppression. Targeting of scavenging receptors could also be explored but its effects in the remaining anti-tumoral scavenging cells could lead to detrimental effects. Inhibition of the aforementioned molecules could also compromise other cellular entities other than TAFs and their mechanisms should be thoroughly evaluated before clinical implementation.

In conclusion, the modulation of the TME presents a promising avenue for immunotherapy development, particularly in highly heterogeneous tumors such as GBM. Synergistic advancements in knowledge, laboratory techniques, data integration, and therapeutical methodologies will continue to be instrumental in overcoming the unique challenges posed by this malignancy, presenting opportunities for the development of more effective and personalized treatment strategies for GBM patients. In this sense, multimodal strategies, such as the combination of ADC-based therapies with ICI or ADCs administration with focused ultrasound for better local delivery, are promising avenues currently being explored by researchers in the field. Regarding the discovery of meaningful target proteins, approaches like the TS-MAP could provide valuable insights into the relationship between membrane dynamics and endocytosis of surface proteins for the discovery of other targetable vulnerabilities of tumor cells. As mentioned earlier, combining the TS-MAP with single-cell proteomics could be a way forward to continue the exploration of how niche-specific adaptations influence the surfaceome landscape. However, the resolution of current proteomic methods and the material amount needed for yielding representative protein profiles is still open for improvement. Also, the integration of high-resolution multi-omics information holds great promise for elucidation of the complex cancer biology, but data integration is a significant challenge due to the heterogeneity of data formats, the sheer volume of information, and the need for robust computational methods to handle complex interactions between omics layers. Nonetheless, these challenges are actively being facilitated by the development of advanced computational tools that can handle diverse data formats, perform high-dimensional analysis, and identify meaningful biological insights.

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# Appendix