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Liljedahl, Emma

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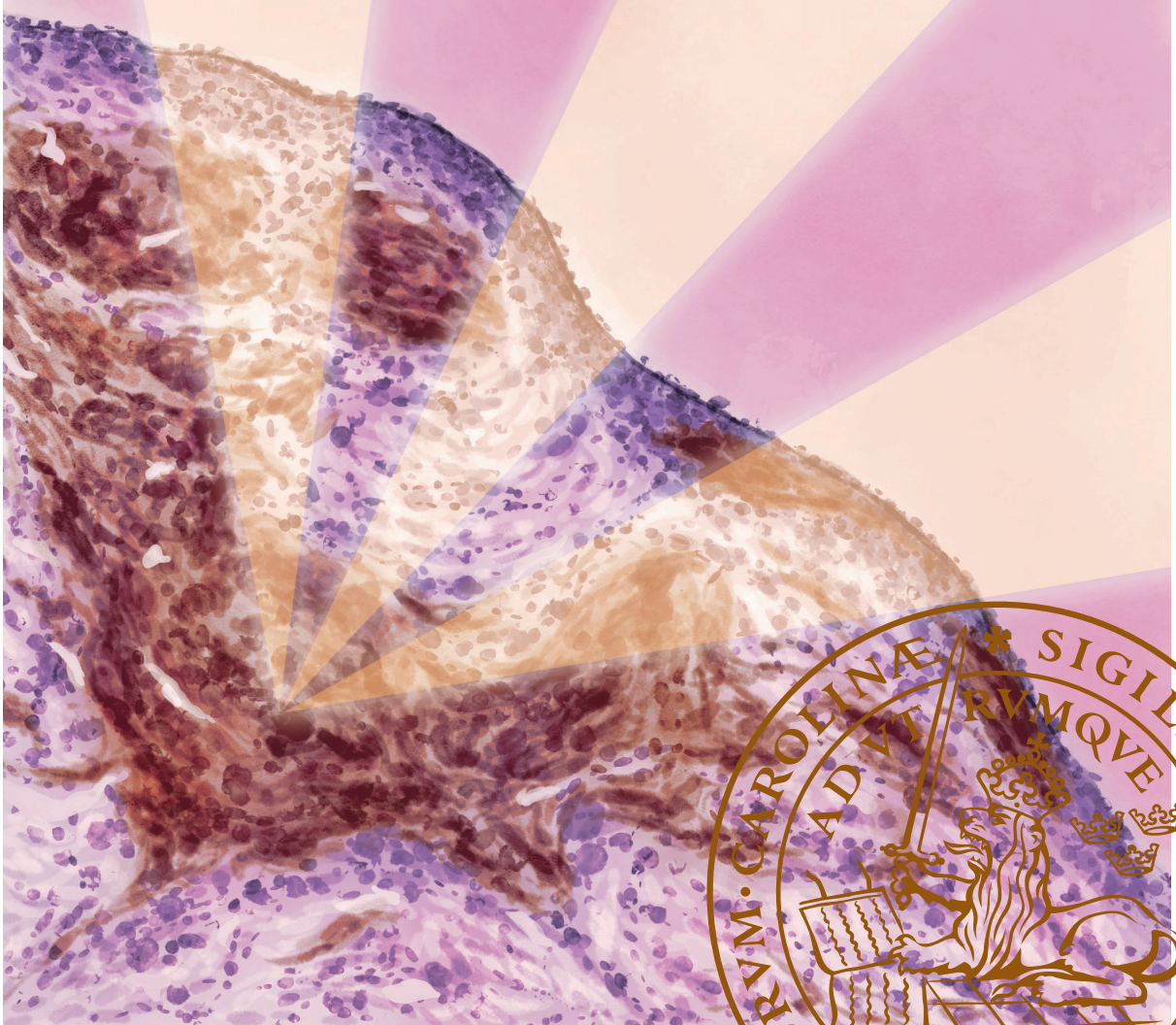
EMMA LILJEDAHL
DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY



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Emma Liljedahl

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 6th of December at 13.00 in Belfragesalen, BMC Lund.

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Abstract:

Glioblastoma is our most common and most fatal primary brain tumor. Although continuous progress in the cancer research-field the prognosis for glioblastoma remains detrimental. Radiotherapy is a corner stone in the treatment arsenal and can induce an immune response, needed to achieve better tumor control. A limitation that remains is the side-effects on healthy tissue. The new implementation of FLASH-RT in the field has in previous studies proven to reduce the unwanted side-effects. It has yet to be extensively explored in fully immunocompetent animals. Also, it is important to establish that it is equal in anti-tumor efficacy compared to conventional radiotherapy (CONV-RT).

Combining radiotherapy and immunotherapy is another treatment option to try and overcome the challenges presented by glioblastoma. Yet, no such treatment is used in the clinical practice. In the present work, we have explored the effects of stimulation of the complement system. The complement system is part of the innate immune system and is swiftly activated. Through its activation it can function as a bridge between innate and adaptive immune responses. We have previously demonstrated that glioblastoma cells inhibit important pathways in the complement cascade to escape immune detection and eradication.

Utilizing a fully immunocompetent Fischer 344 rat model inoculated with glioblastoma cell line in both the intracranial and subcutaneous setting, we set out to explore both FLASH-efficacy and safety and the implementation of radiotherapy with complement activation by using anti-C1-INH antibodies.

We could see that the dose response was similar for CONV-RT and FLASH-RT in our present model. Tumor size upon the time of euthanasia correlated inversely with the irradiation dose. In the intracranial setting survival was prolonged in animals treated with FLASH or CONV-RT, but cure was not reached. CONV-RT and FLASH were equally effective in fully immunocompetent animals with glioblastoma. Radiotherapy was highly efficient in the subcutaneous setting, leading to cure and long-term immunity in the majority of the animals.

Anti-C1-INH treatment could improve the efficacy of irradiation delivered at sub-therapeutic doses and delay tumor growth in the subcutaneous tumor microenvironment. In the intracranial setting, the doses of anti-C1-INH were not enough to achieve any survival effect in the present setting.

Taken together, we have demonstrated that FLASH is equally effective compared to CONV-RT in fully immunocompetent animals with glioblastoma, both in terms of short-term and long-term tumor control.

Key words: Glioblastoma, experimental glioma models, CONV-RT, FLASH, complement system, C1-INH

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Emma Liljedahl

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Faculty of Medicine
Department of Clinical Sciences, Lund

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“You can never be overdressed or overeducated.”

— Oscar Wilde

Till mormor och farmor, jag önskar att ni hade kunnat vara här.

Table of Contents

Abstract

Glioblastoma is our most common and most fatal primary brain tumor. Although continuous progress in the cancer research-field the prognosis for glioblastoma remains detrimental. Radiotherapy is a corner stone in the treatment arsenal and can induce an immune response, needed to achieve better tumor control. A limitation that remains is the side-effects on healthy tissue. The new implementation of FLASH-RT in the field has in previous studies proven to reduce the unwanted side-effects. It has yet to be extensively explored in fully immunocompetent animals. Also, it is important to establish that it is equal in anti-tumor efficacy compared to conventional radiotherapy (CONV-RT).

Combining radiotherapy and immunotherapy is another treatment option to try and overcome the challenges presented by glioblastoma. Yet, no such treatment is used in the clinical practice. In the present work, we have explored the effects of stimulation of the complement system. The complement system is part of the innate immune system and is swiftly activated. Through its activation it can function as a bridge between innate and adaptive immune responses. We have previously demonstrated that glioblastoma cells inhibit important pathways in the complement cascade to escape immune detection and eradication.

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Abbreviations

BBB	Blood-Brain-Barrier
C1-INH	C1-inihibtor
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
CONV	Conventional dose rates
CONV-RT	Conventional Radiotherapy
DR	Discrimination Ratio
FLASH-RT	FLASH Radiotherapy
GAMs	Glioma-Associated Macrophages/Microglias
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GBM	Glioblastoma Multiforme
IDH	Isocitrate Dehydrogenase
IDH-wt	Isocitrate Dehydrogenase - wild type
IDO	Indoleamine-pyrrole 2,3-dioxygenase
ROS	Reactive Oxygen Species
RT	Radiotherapy
TAMs	Tumor-Associated Macrophages/Microglias
TME	Tumor Microenvironment
TMZ	Temozolomide
TTF	Tumor-Treating Fields
UHDR	Ultra-high dose rate

Populärvetenskaplig sammanfattning

Kan "supersnabb" strålning bli framtidens behandling mot hjärncancer?

Föreställ dig en strålbehandling som inte bara är lika effektiv som dagens metoder utan också skonar hjärnan från onödiga skador. Det låter kanske för bra för att vara sant, men den nya strålningstekniken FLASH visar lovande resultat. Hur fungerar denna supersnabba behandling, och kan den verkligen bli ett genombrott i kampen mot hjärntumörer?

Du har varit frisk och levt hälsosamt nästan hela livet. Så plötsligt bär inte ena benet mer och du har svårt att få fram orden. Du söker vård, man gör en röntgen av hjärnan och får svaret misstänkt ”glioblastom”.

Glioblastom? Få har hört ordet förut, detta trots att det är en av våra vanligaste och mest aggressiva typer av hjärntumör. Mycket är fortfarande okänt kring glioblastom och forskarna vet ännu inte varför vissa drabbas. I snitt har den drabbade en överlevnad på endast 8–13 månader.

Det finns i nuläget inget botemedel mot glioblastom, men det finns behandlingar som förlänger överlevnaden, om än fortfarande kortvarigt. Idag är de behandlings-alternativ som finns operation, strålning och cellgifter, ofta i kombination med varandra.

Jämfört med andra cancertyper där det kommit nya revolutionerande behandlingar de senaste åren som förbättrat överlevnaden märkvärt, har man inte funnit någon bot för patienter med glioblastom.

Det skrivs mycket om kroppens immunförsvar. Kanske är det mest bekant att det hjälper till att bekämpa och skydda oss mot bakterier och virus. Men immunförsvaret kan även hjälpa kroppen att bekämpa cancerceller. Skulle ökad kunskap om immunförsvaret kunna bli en del av lösningen?

”Men om vårt immunförsvar kan bekämpa cancercellerna, hur kommer det sig då att cancer ens överhuvudtaget blir ett problem?”. Cancercellerna utvecklar metoder

för att kunna ducka för immunförsvarets radar och har också egna system för att stoppa immunförsvaret.

Vår forskargrupp har valt att studera en del av immunförsvaret som består av enskilda proteiner som samarbetar för att lokalisera och varna resten av immunförsvaret. Detta kallas komplementsystemet. Vi har tidigare beskrivit att glioblastomceller uttrycker ett protein som heter C1-INH, vilket blockerar komplementsystemet. Vi har testat att ge antikroppar mot C1-INH, för att försöka återaktivera vissa delar av immunförsvaret.

Strålbehandling har använts för att behandla cancer sedan förra sekelskiftet, bara några år efter att Wilhelm Röntgen upptäckte röntgenstrålning för första gången. Under lång tid trodde man att det var den direkta strålningen från röntgenstrålarna som gav effekt, men på senare tid har man upptäckt att strålningen gör betydligt mycket mer än så i kampen mot cancer. Genom den direkta skada som strålningen orsakar på cancercellerna hjälper den även immunsystemet. De immunologiska effekterna utav strålningen beror på flera faktorer, däribland den stråldos som ges.

Det finns dessvärre en del biverkningar med strålning. Även om man försöker rikta den mot cancern drabbas ändå friska celler. När man ger strålning mot hjärnan begränsas den stråldos som man kan ge utav risken för skador på frisk vävnad. Dessutom innebär strålning mot glioblastom enligt dagens riktlinjer ofta daglig strålning fem dagar i veckan i sex veckor, eftersom man vill administrera strålningen fraktionerat, det vill säga uppdelat i flera doser.

Vi har därför börjat undersöka en typ av ”supersnabb” strålning som kallas för ”FLASH”. Den ger samma stråldos under några millisekunder jämfört med minuter för vanlig strålning. Andra forskargrupper har i ett mindre antal studier så här långt demonstrerat att man kan minska skador på minnesfunktioner i djurmodeller när man bestrålar med FLASH istället för vanliga formen av bestrålning. I det aktuella forskningsprojektet har vi testat om FLASH är lika effektivt som vanlig bestrålning när det kommer till att döda cancerceller. Det har nämligen funnits en farhåga att om FLASH besparar normala celler i större utsträckning, kanske även cancerceller överlever. Så är dock inte fallet i våra tumörmodeller, där vi sett att FLASH är lika effektivt som vanlig bestrålning och att det också kan ge samma långtidsskydd mot nya cancerceller. Vi har också testat att kombinera strålningen med antikroppen anti-C1-INH, och då kunnat öka den tumördödande effekten.

Det återstår dock fortfarande mycket forskning att göra innan detta kan nå patienten men troligen är det en kombination av flera olika behandlingar som ger oss en lösning.

Original papers

Paper I

Long-term anti-tumor effects following both conventional radiotherapy and FLASH in fully immunocompetent animals with glioblastoma.

Emma Liljedahl, Elise Konradsson, Emma Gustafsson, Karolina Förnvik Jonsson, Jill K. Olofsson, Crister Ceberg & Henrietta Nittby Redebrandt

Sci Rep 12, 12285 (2022). https://doi.org/10.1038/s41598-022-16612-6

Paper II

Comparable Long-Term Tumor Control for Hypofractionated FLASH Versus Conventional Radiation Therapy in an Immunocompetent Rat Glioma Model

Elise Konradsson, Emma Liljedahl, Emma Gustafsson, Gabriel Adrian, Sarah Beyer, Suhayb Ehsaan Ilaahi, Kristoffer Petersson, Crister Ceberg, Henrietta Nittby Redebrandt

Adv Radiat Oncol 2022 Vol. 7 Issue 6 Pages 101011 Accession Number: 36092986 PMCID: PMC9449779 DOI: 10.1016/j.adro.2022.101011

Paper III

Comparable Survival in Animals with Intracranial Glioblastoma Irradiated with single-fraction Conventional Radiotherapy or FLASH Radiotherapy

Emma Liljedahl, Elise Konradsson, Karin Linderfalk, Emma Gustafsson, Kristoffer Petersson, Crister Ceberg, Henrietta Nittby Redebrandt

Front. Oncol., 16 January 2024 Sec. Radiation Oncology Volume 13 - 2023 https://doi.org/10.3389/fonc.2023.1309174

Paper IV

Combined anti-C1-INH and radiotherapy against glioblastoma

Emma Liljedahl, Elise Konradsson, Emma Gustafsson, Karolina Förnvik Jonsson, Jill K. Olofsson, Kurt Osther, Crister Ceberg & Henrietta Nittby Redebrandt

BMC Cancer 23, 106 (2023). https://doi.org/10.1186/s12885-023-10583-1

Papers not included in this thesis

Upregulation of C1-inhibitor in pancreatic cancer

Kurt Osther, Karolina Förnvik, Emma Liljedahl, Leif G. Salford and Henrietta Nittby Redebrandt

Oncotarget. 2019; 10:5703-5712. https://doi.org/10.18632/oncotarget.27191

What is the role of CRP in glioblastoma?

Karolina Förnvik, Aida Maddahi, Emma Liljedahl, Kurt Osther, Leif G. Salford, Henrietta Nittby Redebrandt

Cancer Treatment and Research Communications, Volume 26, 2021,100293, https://doi.org/10.1016/j.ctarc.2020.100293

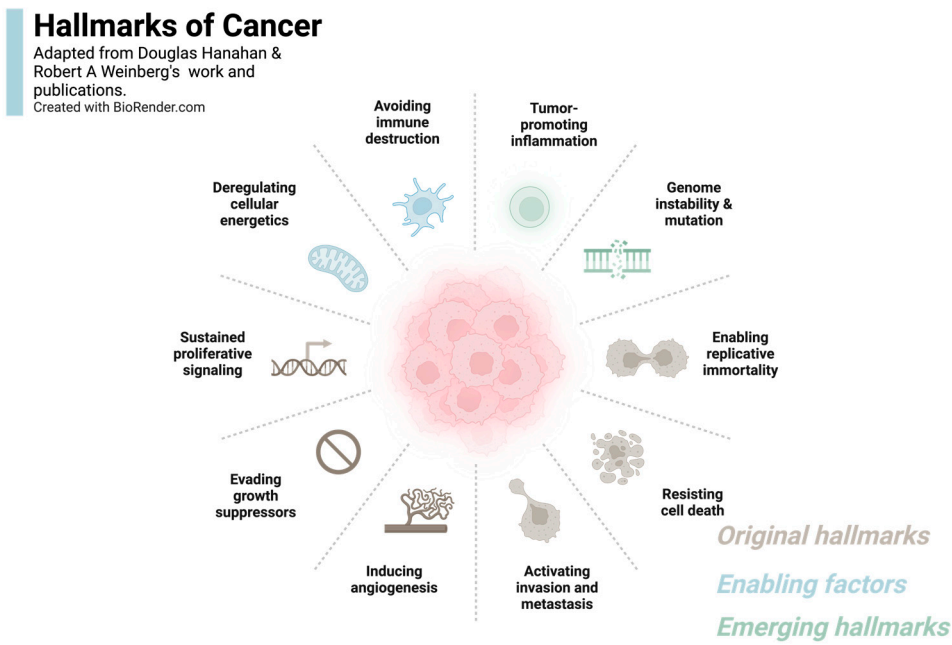
Complement Components in Peripheral Blood from Adult Patients with IDH Wild-Type Glioblastoma

Karolina Förnvik Jonsson, Emma Liljedahl, Kurt Osther, Johan Bengzon, Lillemor Melander Skattum, Henrietta Nittby Redebrandt

World Neurosurgery, Volume 177, 2023, Pages e742-e747, ISSN 1878-8750, https://doi.org/10.1016/j.wneu.2023.06.133

Background

Since 2020, cancer has been the leading cause of death globally, with 10 million deaths accounted for that year. [1] In recent years, however, many previously dire prognoses have been replaced with a more hopeful approach, not least due to the discovery of immunotherapy. To understand this better, I would like to refer to the Hallmarks of Cancer by Hanahan and Weinberg. First described in their 2000 publication, they presented the initial six acquired capabilities of cancer cells [2], Later, these evolved to include an additional four hallmarks and four characteristics, following publications in 2011 and 2022. [3, 4]



The Hallmarks of Cancer serve as a tool to narrow down the now impressive and enormous field of cancer research, which has exploded over the last two decades, into core principles of the evolution from normal cell to cancer cell. [2-4] Among these are the hallmark of inflammation and the ability to avoid immune destruction, which have garnered significant attention in recent years with the development of immunotherapy. In short, compared to conventional cancer treatments,

immunotherapy utilizes the body's own immune system to fight cancer. [2-4] Revolutionary treatments and success stories have emerged in recent years for previously deadly cancer diagnoses, such as malignant melanoma and hematologic cancers. [5, 6] Unfortunately, this progress has yet to be extended to patients diagnosed with aggressive primary brain tumors.

Glioblastoma

In Sweden, there are around 500 new cases each year of glioblastomas, and a global incidence of 10 in 100,000 [7]. However, due to its poor prognosis, it is still a truly devastating diagnosis. Even with all available treatment options and among patients well enough to be able to participate in clinical trials, the median survival is only around 12–15 months. [8]. Most of the patients, however, would not even meet the inclusion criteria of recent clinical phase III trials. [9, 10] According to guidelines, it is recommended that patients with recurrent or progressing glioblastomas are included in clinical studies, and adult patients with newly diagnosed glioblastomas should also be considered. [8]

The current standard treatment protocol by Stupp et al. consists of maximal safe tumor resection followed by radiation therapy (RT) administered as 60 Gy in 30 fractions, 5 days per week, with concomitant and adjuvant temozolomide, a chemotherapy drug.

Despite this aggressive treatment, glioblastoma tumors remain highly treatment resistant. [11, 12]

Origin

One of the very few known contributing factors is previous exposure to radiation, for example patients who have previously received radiotherapy for other cancer types [13]. It is however, also important to point out that these patients already had one cancer diagnosis and no there is no evidence that exposure to routine diagnostic radiation in the clinical setting contributes to the development of GBM [14]. Yet no link to any environmental factors have been made regarding glioblastoma. [15-21]

However, there are genetic syndromes associated with a higher risk of glioblastoma development. These are:

- Neurofibromatosis type 1 (NF1)
    - NF1 is an autosomal dominant genetic disorder and among the most common of these types of hereditary disorders. The product of the

NF1 gene mutation results in RAS hyperfunction, where brainstem gliomas are the 2nd most prevalent intracranial tumor in these patients. [22]

- Li-Fraumeni Syndrome (LFS)
    - LFS results from mutations in the tumor suppressor gene TP53, resulting in an inherited high risk of cancer devolvement. It includes a wide range of different neoplasm from soft tissue sarcomas, leukemias and CNS-tumors. [23]
- Turcot syndrome
    - Patients with Turcot's syndrome are characterized by the occurrence of primary brain tumors along with multiple colorectal adenomas. It seems to stem from two distinct types of mutations, either APC gene mutation or mismatch-repair gene mutation. [24]

Pathways and molecular mechanisms

In the publication by the Cancer Genome Atlas research form 2008, three critical pathways with frequent genetic alterations were presented. [25]

1. RTK/ RAS / PI(3)K, altered in 88% of the cases.
    a. Receptor tyrosine kinases (RTKs), such as EGFR, are amplified in 45% of the cases, leading to RAS activation. RAS proteins are amongst the most common oncogenes in human cancers, regulating cell growth, differentiation and apoptosis.[26] RTKs may signal directly to PI(3)K enzyme, part of the AKT/mTOR pathway, one of the most commonly activated pathways in cancer.[27]
2. p53, altered signaling in 87% of the cases.
    a. p53 is a well-known tumor suppressor gene, and perhaps the most known since its discovery as a suppressor gene in the late 70s, as it is found to be dysfunctional in most cancers. Its dysfunctionality leads to uncontrolled growth and division. [28]
3. RB, altered signaling in 78%
    a. RB1, or retinoblastoma suppressor gene, is also a known tumor suppressor gene. Most known for its involvement, or rather lack thereof, in the development of the pediatric cancer retinoblastoma, it is involved in many other cancer types as well. [29] In the case of GBM, it was mostly altered through alteration in upstream regulators such as CDKN2A (p16), a gene known to encode a multitude of tumor suppressors and mutation and gene loss are related to several tumors. [30]

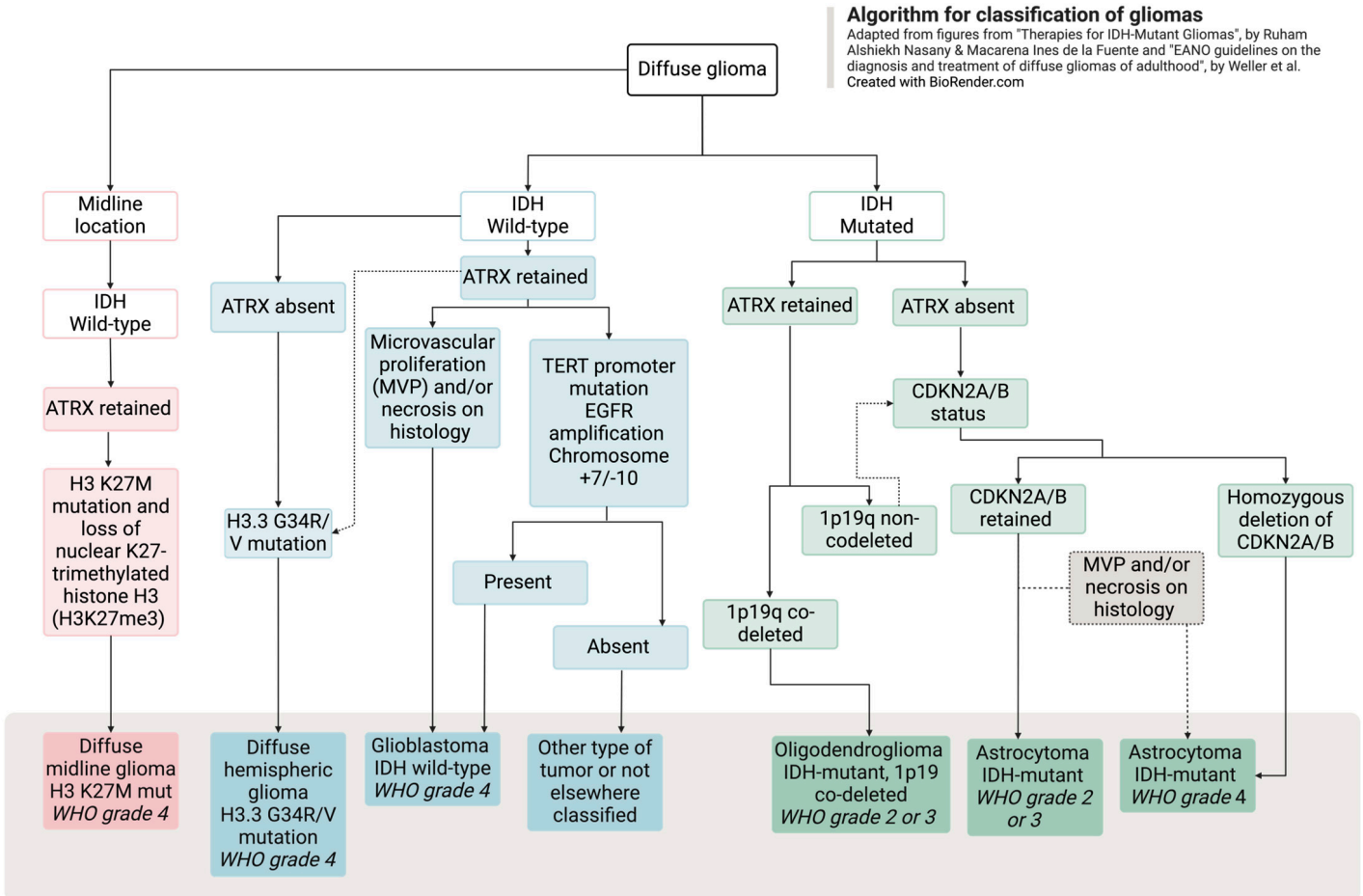
Histology and hallmarks

The World Health Organization published their 5th version of Central nervous system tumors classification in 2021. [31]

World Health Organization Classification of Tumors of the Central Nervous System, fifth edition

- Gliomas, glioneuronal tumors, and neuronal tumors
    - Adult-type diffuse gliomas
        - Astrocytoma, IDH-mutant
        - Oligodendroglioma, IDH-mutant, and 1p/19q-codeleted
        - Glioblastoma, IDH-wildtype

For a more detailed overview please see the figure on the next page, which provides an algorithm adapted from figures from "Therapies for IDH-Mutant Gliomas", by Ruham Alshiekh Nasany & Macarena Ines de la Fuente [32] and "EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood", by Weller et al [33]. Including key diagnostic genes, molecules, pathways, and/or combinations in major primary CNS tumors.


Algorithm for classification of gliomas
Adapted from figures from "Therapies for IDH-Mutant Gliomas", by Ruham Alshiekh Nasany & Macarena Ines de la Fuente and "EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood", by Weller et al.
Created with BioRender.com
Diffuse glioma
Midline location
IDH Wild-type
ATRX retained
H3 K27M mutation and loss of nuclear K27-trimethylated histone H3 (H3K27me3)
Diffuse midline glioma H3 K27M mut WHO grade 4
IDH Wild-type
ATRX absent
H3.3 G34R/V mutation
Diffuse hemispheric glioma H3.3 G34R/V mutation WHO grade 4
ATRX retained
Microvascular proliferation (MVP) and/or necrosis on histology
Glioblastoma IDH wild-type WHO grade 4
TERT promoter mutation EGFR amplification Chromosome +7/-10
Present
Absent
Other type of tumor or not elsewhere classified
IDH Mutated
ATRX retained
1p19q co-deleted
1p19q non-codeleted
Oligodendroglioma IDH-mutant, 1p19 co-deleted WHO grade 2 or 3
ATRX absent
CDKN2A/B status
CDKN2A/B retained
MVP and/or necrosis on histology
Astrocytoma IDH-mutant WHO grade 2 or 3
Homozygous deletion of CDKN2A/B
Astrocytoma IDH-mutant WHO grade 4

Histology

Glioblastoma is known to have a large presence of both necrotic and hypoxic areas, yet it is a highly vascularized tumor. A main feature of glioblastomas is also the local infiltration of the surrounding brain rather than spreading beyond the central nervous system. [34] Typical is also hypercellularity and nuclear atypia.

Epigenetics

In recent years, with acquired knowledge and new methods, epigenetics influence on gliomas has become more apparent and has even come to challenge the use of histological grading and classification. Epigenetics does not alter the actual code or DNA structure, but rather impact which genes are read and expressed. This can be done in several different ways such as DNA-methylation, histone modification, non-coding RNA and chromatin remodelling. [35]

With improved techniques epigenetics have shown to be an integral part of cancer development and classification. [36] Predating the new classification released in 2021 was the publication regarding DNA methylation-based classification of CNS-tumors by Capper et al in 2018. They designed a new tool for classification to improve diagnostic accuracy. [37]

Isocitrate dehydrogenases

IDH for short, is a known catalyst of oxidative decarboxylation of isocitrate producing alpha-ketoglutarate and carbon dioxide, but also reducing cofactors NAD(P)+ to NAD(P)H+. Its different isoforms have important roles in the cell's metabolism. Wild-type IDH1 is known to be overexpressed in most of the primary glioblastoma tumors. Conversely, in secondary glioblastomas, since the updated WHO classification now known as astrocytoma grade 4, oncogenic mutations to IDH1 and IDH2 have been present. The presence of wild-type IDH has been linked to a shorter survival compared to mutant IDH1 and is now a well-used subdivider for glioblastoma versus astrocytoma grade IV. [38, 39]

G-CIMP

Glioma-CpG Island Methylator Phenotype, is one of the specific epigenetic features found in the subgroups of IDH-mutant gliomas. It is divided into to two subgroups, low and high. The so-called G-CIMP low more resemble the IDH-wildtype and primary glioblastomas, associated with worse outcome. [40]

MGMT

MGMT short for O^6-methylguanine-DNA methyltransferase is a DNA repair enzyme. It can antagonize the otherwise toxic effects of alkylating agents such as Cisplatin and Lomustine amongst others. Methylation of the MGMT promoter is known to silence the MGMT gene and is associated with a favourable outcome in

patients with glioblastoma when receiving chemotherapy with alkylating agents. It is now a standard in the analysis of glioblastoma patients. [41]

Chromosome 7/10

Gain of chromosome 7 and chromosome 10 loss, also known as trisomy 7 and monosomy 10, accounts for one of the most prevalent genomic alternations in GBM's. It is associated with both downregulation of tumor suppressor genes as well as upregulation of oncogenes, for example EGFR. [42]

EGFR

The amplification of the EGFR gene seems to be present in over half of all the patients diagnosed with primary glioblastoma according to Brennan et al's comprehensive work on the glioblastoma genome utilizing the Cancer Genome Atlas Program. [43] Short for epidermal growth factor receptor, EGFR, belongs to the family of receptor tyrosine kinases, RTKs. RTKs account for integral parts in cellular growth as well as differentiation and are known contributors to oncogenesis when altered. [44] EGFR is responsible for a number of different pathways, where the most studied remains to be the pathway including phosphatidylinositol-3-kinase (PI3K), resulting in activation of important proteins for tumor growth, such as AKT and mTOR. [45] All of this has made EGFR an interesting target in anti-tumor treatment in both GBM as well as in other cancers. Unfortunately, although seeming so promising theoretically, these targeted treatments has yet to be proven effective in the battle against the illusive GBM cells. Although a majority of the GBM cells express EGFR, it has also been proven that GBM are able to compensate with activation of other pathways, independent of EGFR signalling. [46-48]

TERT

Telomerase Reverse Transcriptase promoter was originally found when researching melanoma tumors [49, 50]. Telomerase is in charge for the repair of telomeres, an integral part of the chromosome's protection and maintenance. With each cell cycle the telomeres shorten and finally cause cell death, this makes it easy to understand that overcoming shortening is essential for a cancer cells proliferation and has been a subject of interest in understanding cancer development. [51, 52]

Tumor microenvironment

The tumor microenvironment (TME) has gained a lot of attraction in recent studies of cancer. In many forms of cancer, the tumor microenvironment consists of cancer cells, cancer stem cells and a variety of so-called stromal cells. In the case of GBM these stromal cells mainly involve normal or reactive astrocytes, microglia, macrophages, pericytes, fibroblasts and endothelial cells. Together all these individual cells create a multifaceted signalling system of different molecular signals, playing an integral part

in both tumorigenesis and malignant progression by promoting changes in the epigenome benefiting the cancer cells for proliferative expansion. [53] [4] The microenvironment can be divided into different anatomical parts also called *tumor niches* [54], further research suggest that these niches provide the glioma stem cells with the opportunities needed to develop both chemo- and radioresistance. [55] The TME in GBM is known to be "cold" or immunosuppressive, making it less susceptible to immune response elicited from some antitumor treatments. [56]

Cancer stem cells

Cancer stem cells, sometimes called tumor initiating cells, has since their first major introduction 30 years ago gained vast interest within the cancer research field. They are basically tumor cells with the ability to self-renew and with a potential for further differentiation but also with the expression of stem cell markers. They have been seen in several tumor types. Within the GBM field, the existence of cancer stem cells has been debated, but it is clear that some tumor cells are extremely resistant to therapies. [57, 58] This might be one of the underlying mechanisms for GBM's tumor heterogeneity and therapy-resistance.

Hypoxia

Hypoxia is known to stimulate a more immuno-suppressive microenvironment in glioblastomas. [59] It is also very common in large and rapid growing tumors due to the lack of vessel supplying oxygen to the entirety of the tumor. [60] Hypoxia is therefore also a hallmark of GBM, known for its necrotic core, which can be seen both macroscopically and microscopically. [61] Hypoxia-inducible factors, also known as HIFs, were shown to play an important role in modifying the glioma stem cells and promote tumorigenesis as well as activating angiogenesis and invasion through upregulation of several genes. [60, 62] Hypoxia is also a known factor to radioresistance, discussed more in detail later.

Reprogramming of energy metabolism

The reprogramming of energy metabolism was presented as an emerging hallmark of cancer in 2011 by Hanahan and Weinberg, explaining the need of corresponding alternations in the cell metabolism to fuel the excessive growth and division of the cell. [3] Of great interest in the case of GBM is that several studies point to that these metabolic alternations may also play a part in the resistance to chemotherapy, providing new promising targets for the future. Olivia et al first showed from their studies in vitro from 2010 that acquired chemoresistance to the most used chemotherapy for GBM, Temozolomide (TMZ), may be due to a mitochondrial adaptive response to the genotoxic stress caused by TMZ. [63] In following studies, the group's findings suggest a mitochondrial ROS-dependent mechanism underlying TMZ-chemoresistance in glioma. Continuing they showed promising results in 2017 when using chlorpromazine to inhibit cytochrome c oxidase activity from chemoresistant GBM cells. [64-66]

Reactive oxygen species

Reactive oxygen species (ROS), are radicals, ions or molecules with an unpaired electron, leading to its main characteristic of being highly reactive. Cancer cells are known for having high levels of ROS, one of the contributing factors are the reprogramming of energy metabolism in the cell mentioned above which leads to mitochondrial dysfunction, increased oxidase activity, peroxisome activity and overall increased metabolic activity. [67] In the normal healthy cell ROS are continuously cleared from the cell to avoid damage caused by ROS and its reactivity. Cancer cells are known to not have the same effective systems for removal, thus also contributing to the greater level of ROS further adding to the oxidative stress in the cell. [68] The oxidative stress alters signalling pathways and cellular behaviour further and have been linked to several changes in the cancer cells from metabolism to angiogenesis and stemness, further promoting tumor progression and the oncogenic phenotype. [67, 69-71]

Tumor infiltrating immune cells

GBM is known to be an immunologically cold tumor, which simply means that it does not elicit a strong T-cells response and thus have few infiltrating lymphocytes. [72]

A key component seen in the malignant gliomas are infiltration by tumor-associated, also known as glioma-associated macrophages or microglia (TAMs or GAMs for short). They are highly prevalent in the tumor and in some samples, they are seen to represent up to 50% of the cells seen in human GBM. [73, 74] They have been associated with negative prognostic factors such as increased invasiveness and also increased tumor growth. [75, 76]

TAMs are comprised of both macrophages and microglia and although these might be morphologically different it has been shown that they can sometimes share similar markers making this a more complicated target than anticipated. [77, 78]

Additionally, they are also usually divided into two different phenotypes, M1 and M2, where M1 is known as the classic and pro-inflammatory phenotype and M2 is called the alternatively activated and anti-inflammatory phenotype. M2 is divided into 3 additional subtypes with different functions. M2a, responsible for Th2 response, type II inflammation, allergy and killing of pathogens. M2b associated with Th2 activation and immunoregulation, M2c with immunoregulation, matrix deposition and tissue remodelling. [79]

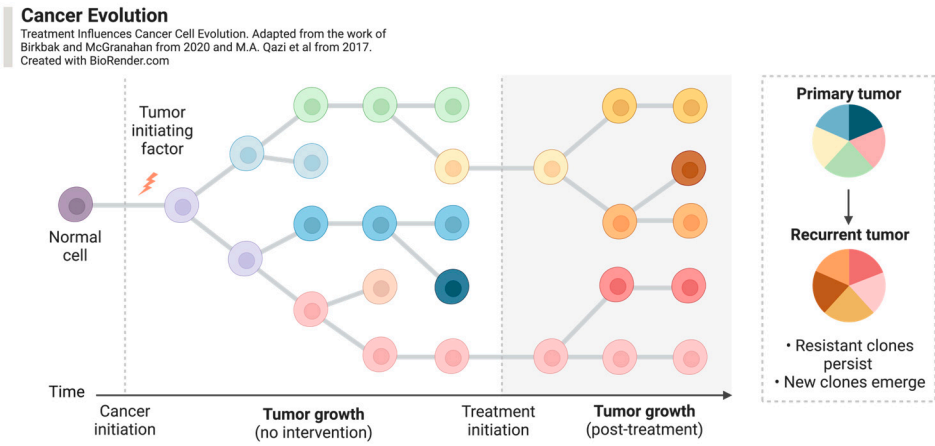
Apart from TAMs and GAMs myeloid-derived suppressor cells (MDSCs) are another commonly found cell in the GBM TME, the cells consist of immature myeloid precursors, and all contribute to immune suppression via a number of different mechanisms. The glioma cells seem to recruit both GAMs and MDSCs to the tumor site and then induce an immunosuppressive phenotype in these cells, contributing to the immunosuppressive nature of the TME. But they also act in a tumor-promoting manner, stimulating tumor growth, invasiveness and neovascularization. [80]

Tumoral heterogeneity

It is now a well-established fact that malignant tumors exhibits both an intertumoral as well as an intratumoral heterogeneity. [81, 82] Glioblastoma has excelled as one of the most heterogenous tumor types, making it highly illusive. Its combinations of not only a variety of differentiated cells, glioma stem cells, a complex tumor microenvironment and not at least it's often vastly different histomorphology giving rise to the "multiforme" aspect of the tumor. [83] Tumoral heterogeneity is basically the existence of several different clones existing within the same tumoral disease, these clones all have varying molecular and genetical characteristics. Intertumoral heterogeneity is this above stated variation found in the different tumors of the same origin cancer in the patient. Intratumoral heterogeneity however is the presence of these variation within the same solid tumor.

Patel et al performed single-cell RNA sequencing on GBM from five different patients showing that these 430 cells were all inherently different. Showing diverse transcriptional programs related to oncogenic signalling, proliferation, hypoxia as well as both complement and immune response. Proving that in order to better understand glioblastoma these are critical findings needed to develop therapies that address the complexity of this disease. [84] Soeda et al showed that GBM subclones from a patient had the ability to, in both in vitro and in vivo, produce heterogenous tumor cells with self-renewal. [85] The glioma stem cells or initiating cells are known contributors to the tumor heterogeneity of GBM. [86] But the heterogeneity is also dynamic, which might better explain the continued resistance to even combined therapies. Kim et al performed a whole-genome and multisector exome sequencing of primary and post-treatment GBM, genetic alteration of p53 was predictive of a high number of subclonal mutations in glioblastoma. Overall, it provided new insight into how different genetic alterations in the primary tumor influence the consequent evolution and subclonal heterogeneity. [87] In 2016 Wang et al published their study where they analysed genomic and transcriptomic data from GBM patients through therapy. They found that 63% of the patients had an expression-based subtype change during the analysis. Proving further that the evolution of GBM when faced with treatment was a complex process with its own precise changes and evolutionary patterns. Of particular interest was that at the time of relapse nine patients had lost EGFRvIII, since EGFR has been a promising target for therapy these finding once again highlights the difficulties in treating a highly heterogenic tumor such as GBM. [88] Johnson et al found in their study of low-grade gliomas that the primary and recurrent tumor only shared a few similar early mutations. In 43% of the reported GBM cases, almost half of the mutations found in the initial tumor were now unobserved at recurrence, further strengthening the theory that exposure to therapy greatly influence the evolution of the tumor cells. [89] Noeroexe et al studied GBM patients that had undergone relapse surgery and found differences in mutations dynamics between the patients but also that driver mutations varied during progression of the tumor. [90]

Adapted from the work of Birkbak and McGranahan from 2020 and M.A. Qazi et al from 2017, below is a figure with a schematic overview of the suggested evolution of the subclones in response to treatment. [91, 92]



As Qazi et al points out in their review *"Current models for the study of GBM fail to directly address the problem of GBM recurrence and continue to focus efforts on understanding primary, treatment-naïve tumor biology."*.

Tumoral heterogeneity adds to the challenge in treating all cancer types, for example biopsy from a primary tumor may have completely different characteristics compared to a metastasis of that same tumor. How does this affect the response to treatment, since this is often based on information given by the analysis of said biopsy? In GBM the intratumoral heterogeneity is perhaps one of the most important aspects in understanding its treatment resistance. [86]

A brief overview of the immune system in cancer

The immune system is built up by the innate and the adaptive immune system. The first initial response to foreign entities is the innate immune system, acting as the first line of defense. Put in very broad and simple terms the innate immune system consists of physical barriers as well as small bioactive molecules, compared to the adaptive immune system of B-cells and T-cells with their antigen-specific-receptors, making this second-line defense one of more target seeking. [93, 94]

The CNS immune system

The CNS immune privilege

Going back to the first half of the 20th century, studies where one tried to transplant tissue into to brain showed, compared to when transplanted to the rest of the body, a lack of rejection to the new tissue. Thus, this gave rise to the knowledge of the brain's unique immunological response. [95]

The blood brain barrier

One of the main structures responsible for the unique and isolated nature of the CNS immune system is the blood-brain-barrier (BBB). Lining the delicate capillaries in the CNS are endothelial cells, these are in turn surrounded by:

- Pericytes, contractile cells
- Astrocytes, initially thought to only be part of the structure of the CNS, it is now well established that astrocytes are integral cells in both homeostasis and immune response. [96]
- Microglia, the central nervous system's own immune cell. It is now also believed to serve other purposes in the healthy brain. [97]

Between each endothelial cell lays the tight junctions, connecting the cells, and creating the barrier which only allows certain molecules entrance, generally inhibiting those larger than 400Da or water-soluble, for example this limits most antibody-dependent treatments [98].This delicate structure covers most vascular parts of the brain, exceptions are the choroid plexus and circumventricular organs. Both primary and metastatic tumors affect the BBB. In gliomas this disruption of the BBB varies depending on the stage of the disease, but it is often most prevalent around the tumor itself, yet it is not that simple as it has also become apparent that large areas still present an intact BBB. [99]

The BBB disruption is caused by the often immature and more permeable vessels of highly malignant tumors such as glioblastoma. [100, 101]

Having its obvious advantages in the defense against intruders outside the CNS it is also a great obstacle in the treatment of brain tumors. [100]

The glymphatic system

It is now well established that classical lymphatic vessels are present in the meninges as well as immune cells. [102] This was discovered during the late 20th century, and it was also established that CSF could drain to cervical lymph nodes and that T-cells could enter the CNS. This could be explained when further knowledge regarding the crossing of CSF with brain interstitial fluid (IFS). [103-106] The glymphatic system is a clearance system of perivascular channels formed by astroglial cells. Not only is it part of the CNS elimination system but might also

have functions related to allocation of necessary compounds to the CNS. Interestingly it seems to be mainly active during sleep. [107] It was first characterized in vivo as late as in 2012 in mice by Iliff et al. [108] Hadaczek et al studied convection-enhanced delivery of viruses, fluorescent liposomes or bovine serum albumin in rats. Their findings supported that the circulation within the CNS through the perivascular space is the primary mechanism by which therapeutic agents, and substances alike, administered by convection-enhanced delivery spread throughout the brain. This prompts speculation as to whether the glymphatic system might be a possible route for delivery of cancer drugs to the CNS. [109]

The complement system

The complement system provides a bridge between the innate and the adaptive immune system.

It is also involved in maintaining homeostasis by detecting and responding to pathogens and altered self. It can be activated by different molecular structures, including antibodies, which initiate a proteolytic cascade marking cells for destruction.

Activation of the complement can be achieved through three different pathways known as; the classical, the lectin mediated, and the alternative pathway. [110]

The classical pathway and C1-inhibitor

The C1 complex consists of subcomponents; C1q, C1r and C1s. When C1q binds to an antibody this causes a dissociation of C1-INH from pro-C1 and allowing the autocatalytic cleavage to proceed. C1-INH then binds covalently to the active sites on C1r and C1s, inactivating their catalytic function and dissociating them from C1q. This bond is irreversible and therefore prevents cleavage of C4 and controls the initial amplification step of classical-pathway activation. [111]

Thus, C1-INH is an efficient inhibitor of the classical pathway of the complement system[112, 113].

Schematic simplified overview of the classical pathway in the complement system and the role of C1-INH

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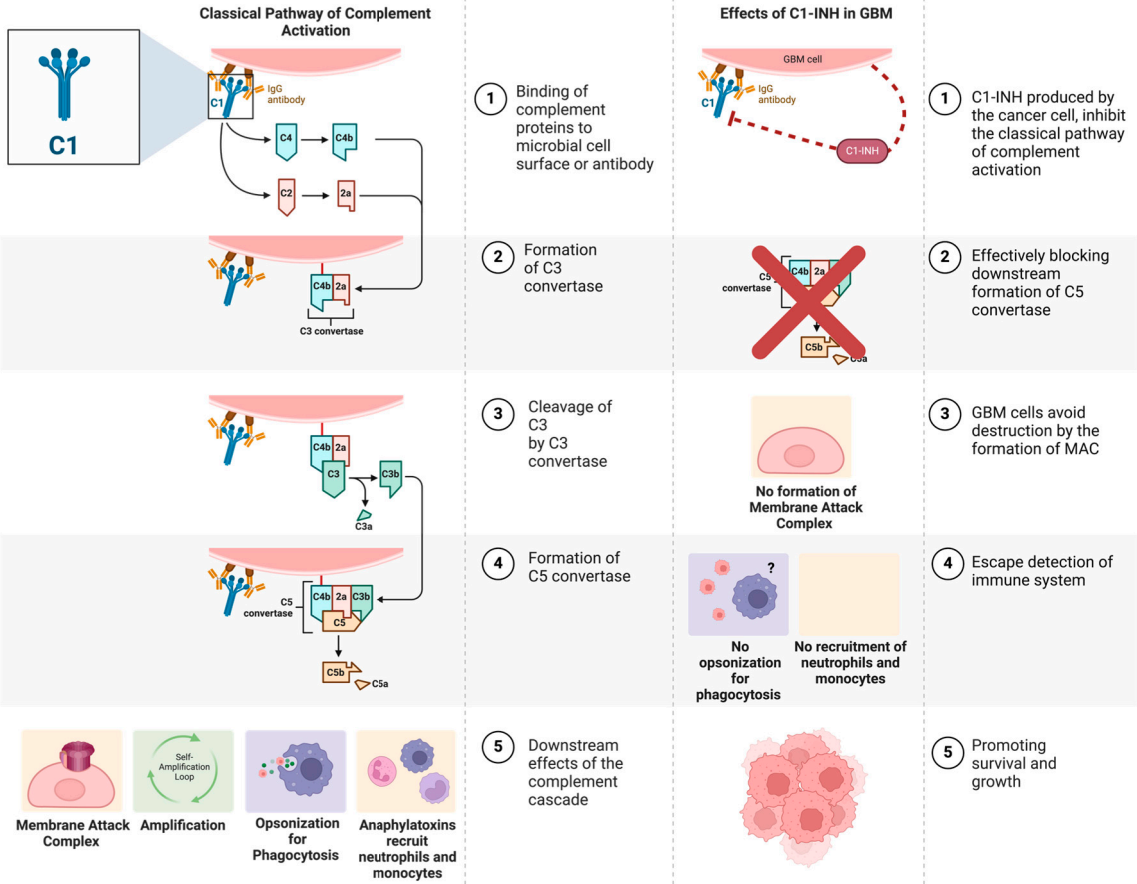


Apart from this C1-INH also limits several proteases of the coagulation and anticoagulation system, including factor XI, factor XII, plasma kallikrein, plasmin, and tissue plasminogen activator [111, 114].

Hereditary angioedema (HAE) is caused by the genetic deficiency of the C1-INH and is transmitted as an autosomal dominant trait [112].

C1-INH has also been linked to inhibit the acute rejection after transplantation in animal models, however, little is known how this reflects in the human settings [114].

In CNS, both astrocytes and microglia are responsible for some of the early components in the complement cascade. Astrocytes are well known to produce C3, C4 and C1-INH. [115, 116] Microglia, apart from producing C1q when exposed to proinflammatory cytokines such as IFN-y or IL-1b, also expresses complement receptors. Neurons in turn produces C1-INH to protect themselves from complement attack. [117, 118]

It has also been shown that the complement system is involved in several parts of the neurodevelopment. Gorelik et al showed that C3 knockdown resulted in incomplete neural migration, which has been linked to schizophrenia. [119] C1-INH also contribute both migration as well as proliferation in the prenatal stages. [120]

In neurological disorders C1q have been linked to Alzheimer's disease, where it seems to at least contribute to the exclusion of microglial synapses and anti-C1q could inhibit these changes. [121]C3 and C5a also have important parts in the development of Alzheimer's disease. Increases in C1q and C3 where seen in amyotrophic lateral sclerosis, ALS. Increased immunoreactivity of several complement proteins was also present in brain of patients suffering from Huntington's disease. [122]

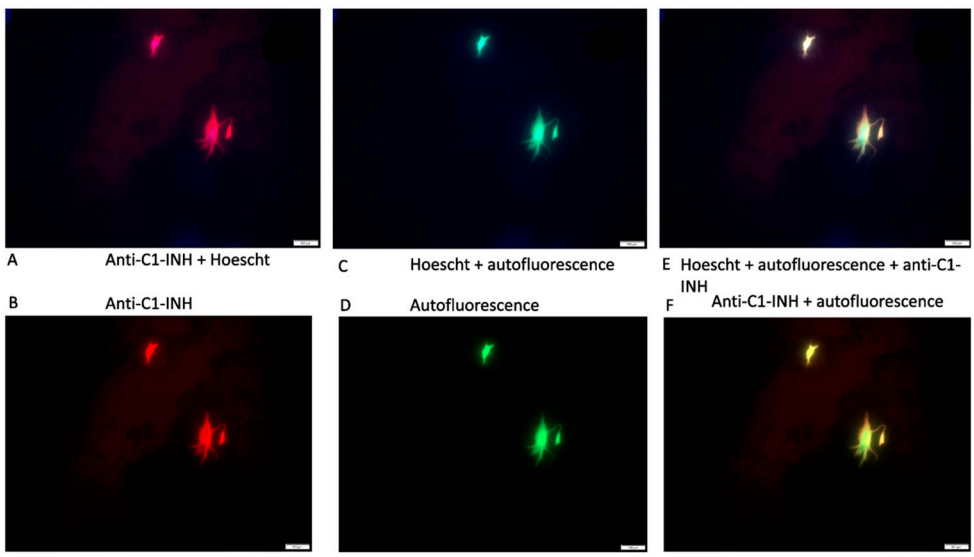
In the CNS setting, Weiss E. et al showed in their article that treatment with C1-INH might reduce the effects of brain oedema due to traumatic brain injury in a rat model. In a pre-clinical setting, C1-INH was administered intravenously in the same session as a traumatic injury was inflicted upon rats, and after 48 h the brains were weighted before and after heating to establish the degree of oedema. C3a levels, which can reflect complement activation, in the brains were reduced in animals treated with C1-INH. [123]

The role of the complement system in cancer is complex as discussed by Revel et al in their 2020 publication. A low degree of activation seems to promote tumor progression while a more potent activation has antitumor effects [124]. Deposits of complement components have been documented in several human tumors suggesting a potential involvement of the complement system in tumor immune surveillance. The lectin pathway has also been implicated in complement activation on glioma cells which express, like many other malignant cells, high mannose glycopeptides that bind MBL and trigger consumption of C4 and C3, but this reaction fails to induce cell lysis[125].

Mediated by C1 activation, treatment with monoclonal antibodies against tumor associated antigens can lead to complement dependent cytotoxicity by formation of the so-called membrane attack complex (MAC), which eventually leads to cell lysis. However, the efficacy of many antibody-based immunotherapies is compromised by regulators of the complement system, whose role is to protect the host from unspecific complement activation. CD59 inhibits MAC formation by binding to C8 and C9 and is highly expressed in many cancer forms. Blocking CD59 resulted in improved treatment efficacy in studies on lung cancer and lymphoma. [126]

Another example is PTXA3, that interacts with C1q and factor H to modulate complement activation. In PTXA3 deficient mouse models, a constant state of complement mediated inflammation was present, favoring skin carcinoma, whereas high levels of PTXA3 in human cancer was associated with shorter survival. [124]

Our previous research, by Förnvik et al from 2017, has demonstrated that C1-INH is upregulated in glioblastoma, both in human tumors and in rat tumor cells. [127]



Anti-C1-INH expression (rabbit anti-rat Covance) demonstrated on NS1 cells in red. NS1 autofluorescence in green and nuclear staining with Hoechst. Scale 1:100 µm in figures A-F. Published in BMC Cancer with our article "Combined anti-C1-INH and radiotherapy against glioblastoma".

And treatment with anti C1-INH in the subcutaneous model could increase survival in fully immunocompetent rats with inoculated glioblastoma [128]. We have previously, in unpublished trials, tried this in the intracranial model as well but with no cure. GM-CSF and IL-1b were decreased in serum from animals treated with anti-C1-INH, but the exact mechanistic steps leading to increased survival in the animals has not yet been demonstrated [128]. However, it is known from other studies that any damage to the brain can increase GM-CSF [129]. However, it is known from other

studies that any damage to the brain can increase GM-CSF [129]. GM-CSF seems to have dual roles in the context of cancer, stimulating dendritic cells and macrophage activity for example, but also promoting tumor growth and metastases [130].

Treatment regime for glioblastoma today

The current treatment options include surgery, radiotherapy, and chemotherapy. Based on EANO Guidelines from 2014, depending on the patients age and performance status, surgery is the first approach, either resection when possible or biopsy. An important aspect regarding surgery is to perform maximal safe resection. There are tools available to use during the resection, such as the usage of fluorescent dye 5-aminolevulinic acid (ALA) to visualize glioblastoma tissue during microsurgical tumor resection. Using 5-ALA has increased the rate of gross total resection and an increased progression-free survival [131]. This in combination with MRI led navigation has greatly improved the possibility of gross total resections while minimizing neurological deficits.

Radiotherapy

Since its first publication in 2005, the Stupp protocol has been the golden standard for patients with histologically confirmed glioblastoma and remains one of the cornerstones in the treatment arsenal. According to the Stupp protocol, radiotherapy is given fractionated with a total dose of 60 Gy, 2 Gy / fraction five days of the week for a total of six weeks. The patients also receive concomitant chemotherapy with Temozolomide given every day of the week during ongoing radiotherapy. Patients can then also receive adjuvant temozolomide in its usual 5-day schedule every 28 days. The Stupp protocol showed that the addition of concomitant and adjuvant temozolomide to radiotherapy significantly improved patient survival. [8, 10]

Radiotherapy (RT) is one of the few treatment options that has led to increased survival in patients with glioblastoma, with a doubled survival time in irradiated patients compared to those who had surgery as stand-alone therapy [12]. However, the dose of irradiation is limited by unwanted side effects on normal tissue. In the brain, normal tissue damage is especially problematic, not least due to cognitive side effects. In order to reduce side effects, RT is usually delivered fractionated over a period of several weeks. [132, 133]. Still, survival is very short in patients with glioblastoma, with a median survival of just 12 months[9].

The combination of radiotherapy and immunotherapy is already a research area of constant news with many clinical trials ongoing[134]. It is of most interest to determine what dose, time difference and fractionation that gives the most favourable outcome. [135, 136]

Previous studies by our group [137] , as well as Formenti et al[135], demonstrated that when radiotherapy is delivered at this optimal dose and fractionation it can indeed induce an effective response from the immune system that may operate in a synergetic manner with immunotherapy. In studies done previously by our group, utilizing IDO inhibition as immunotherapy, the data suggested that radiotherapy should be given in fewer fractions, from 2 to 6 but maintaining larger doses, around 6-8 Gy in rodent models, to favour an optimal immunological response in relation to combination with immunotherapy. [137, 138]

Radiation and neurotoxicity

A reoccurring limitation of the radiotherapy is its neurotoxicity and other side-effects [139], limiting the dosages and treatment period. It was believed up until the 70s that the brain was radioresistant, it would later be shown that acute CNS syndrome post-irradiation occurs after a single dose above 30Gy, with extreme symptoms minutes to hours after exposure and in worst case death. [140, 141]

Whole brain irradiation in young rats resulted in chronic and progressive cognitive impairment up to one year, as demonstrated with novel object recognition task. [142] High singular dose rates has been linked with oligodendrocyte decline is rats [143], whereas fractionated delivery similar to the current treatment regime showed maintained white matter morphology although the rats showed signs of cognitive decline [144]. The mechanisms behind the cognitive decline in patients is not described by one single pathological mechanism, but instead by several different pathways combined.[145] Irradiation seems to affect the neural precursor differentiation, and a main factor deemed to be important is the radiation-induced decrease of neurogenesis. It is important to note that the neurogenesis in the adult brain is limited and therefore its decrease is not the entire mechanism behind the neurocognitive decline following radiotherapy. [146-149] One known alteration that occur in response to radiation is changes in the morphology and physiological functions of dendritic cells. Changes in the spine of the dendritic cells correlates with changes in synaptic function and plasticity and have been observed post-radiation. [150, 151] Irradiation causes mitotic death in endothelial cells, this leads to thrombosis by platelet adhesion exposed to the matrix. This thrombosis affects the small vessels in the brain and hurt the blood-brain barrier, leading to both ischemia as well as neurotoxicity. [152]

Schmal et al explored DNA repair capacities in mice irradiated with low-dose radiation and assessed the effect on hippocampal neurons. They saw that the accumulation of DNA damage led to a decline of hippocampal neurogenesis. Their findings suggested that repeated low-dose radiation impairs neurogenesis. [146] It has previously been stated that a main factor behind radiation induced neuro-deficiency is the vulnerability to ionizing radiation in hippocampus. [153]

Unfortunately, to increase the absorbed dose to a therapeutic level for brain tumors often ends in major neurotoxicity. Any modulation that could advance the therapeutic response without jeopardizing normal tissue tolerance would be instrumental in the strive to improve tumor control. Techniques such as stereotactic radiosurgery and hypofractionated RT are some strategies that may provide this patient group with shorter treatment courses compared to the current Stupp protocol, this is of much importance due to the patient groups' often limited time. [154] Of note is the occurrence of radionecrosis following stereotactic radiosurgery and it is one of the main limitations regarding doses. Although radionecrosis is a known side effect of all cranial radiotherapy, it seems to be more common following stereotactic radiosurgery. [155, 156]

Measuring or analyzing synaptic markers has been used by many to study the changes in neurobiology in rodents post-irradiation. [157-159] An important marker for synaptic plasticity is Synaptophysin that is present in the presynaptic dendrites' membrane. [157] Alaghband et al saw a significant decrease in Synpatophysin density after CONV-RT in the hippocampus, this was however not seen in the rodents irradiated with FLASH-RT. [158] Another commonly used marker is postsynaptic homer scaffold protein 1a or Homer1a. [159]

The inflammatory response after irradiation in relation to neurotoxicity is an important measurement. Acharya et al showed the strong correlation of pro-inflammatory genes and persistent microglial activation post-irradiation, and elimination of the microglia improved cognitive function. [160]Commonly used microglial markers are CD68 and Iba-1. CD68 is upregulated in active microglia and less prominent in resting microglia. [161, 162] Elevated levels of Iba-1 is also a known marker for microglial activation. [163] Toll-like receptor 4 (TLR4) is also a known trigger for proinflammatory response [164]. Alaghband et al could see that FLASH-RT did in fact not elicit the same pro-inflammatory response with elevated levels of CD68, Iba-1 and TLR4 as CONV-RT, suggesting that FLASH-RT provides less of an inflammatory response and thus spares the test subject from cognitive impairment correlated with CONV-RT. [158]

Radioresistance

Still, glioblastoma unfortunately carry both inherent radioresistance and can develop adaptive radioresistance. [165] As described by Boustani et al's in 2019, the 6Rs of radiobiology are used to explain the effect of fractionation and treatment efficacy. These 6Rs are known as the following;

- Repair
- Redistribution
- Reoxygenation
- Repopulation

- Intrinsic Radiosensitivity
- Reactivation of anti-tumor immune response [166]

Ionizing radiation causes harm to the cells partly by directly causing strand breaks in DNA, leading to tumor cell death, but also by water radiolysis, the disassociation of water molecules, this leads to the formation of ROS which in turn causes cell damage through covalent changes in macromolecular parts of the cell.[167] However, the formation of ROS is dependent on oxygen presences in the environment. [168] Oxygen molecules is also known to form peroxides together with the damaged DNA and this inhibits any attempt of reparation. [169] Which of course means that hypoxia contributes to a resistance of these damages, thus, causing radioresistence.

In 2014 Marampon et al showed that inhibition of HIF-1α dramatically improved GBM cells radiosensitivity, proving HIF-1α to be an important target in future research. [170]

IDH is known to produce NADPH, NADPH helps maintaining redox homeostasis, protecting the tumor cells from oxidative stress [39] and thus, enabling radioresistance. Wahl et al showed that by silencing IDH, improved response to radiotherapy was seen in a murine model. [171]

Of interest in the setting of GBM radioresistance is also MicroRNAs. These non-coding RNAs are part of gene expression inhibition and major players in the regulation of stem-like features. They have also been studied in the aspects of being biomarkers. [172] They even show promise as targets in increasing radiosensitivity, Denbg et al showed that miR-124 targeted CDK4. CDK4 is known to in elevated levels contribute to radioresistance and poor prognosis in patients. Even though the complete mechanism to this is still unknown, miR-124 restoration proved that CDK4 was one of the downstream targets, and restored radiosensitivity. [173] Moskwa et al saw that others induced radioresistance in GBM by upregulation of checkpoint response in the cell-cycle. [174]

Radiotherapy and the immune system

Radiotherapy is generally seen as a local treatment, but it is now well established that radiation also has immunomodulatory effects, which can be explored in combination with immunotherapy done by Formenti SC et al but also by our own group in 2020 by Ahlstedt et al with IDO1-INH [135, 137].

DNA damage caused by irradiation is one of the main lethal effects on cancer. An indirect damage is caused by the production of free radicals. Cell death induced by irradiation leads to the release of cytokines, chemokines and tumor antigens and the immune mediated effects of irradiation can be caused by modulation of the tumor microenvironment and the tumor phenotypes. [175]

According to previous research by our group [137], as well as by others [138, 175-177] , radiotherapy delivered at optimal doses and fractions can induce an effective immune response. This appears to create a synergistic effect together with immunotherapy.

Lymphopenia is known negative prognostic factor in GBM, it is unfortunately also a known side-effect of radiotherapy [178], even when said radiotherapy is only given as partial brain RT it has shown to still contribute to systemic lymphopenia. A reason for this might be the large volume of blood passing through the brain continuously.[176] This is especially important to take into consideration when treating GBM as the concomitant treatment with chemotherapy, which is also known to diminish lymphocyte count. [179-182] RT delivered with multiple fractions has been shown to decrease lymphocyte count in circulating blood, but, decreasing the amount of fractions and increasing the dose in each fraction, has been suggested to reduce lymphopenia [176]. In the pre-clinical setting this has been demonstrated that hypo-fractioned high doses result in an increased pro-immunogenic effect [175, 183].

More specifically, it has been demonstrated that radiotherapy can promote T-cell specific immune cell response[177, 184]. The efficacy within the intracranial setting remains to be demonstrated further.

Corticosteroid's effect also needs to be taken into consideration in the treatment of patients with brain tumors, as it is a most common medication to combat the brain edema. Previous research has shown that the combination of RT with ongoing treatment of corticosteroids led to a drastic suppression of the immune system. [185] A long-lasting suppression of the immune system was also seen when patients were treated in accordance with the Stupp protocol with concomitant as well as adjuvant TMZ. Even at the one-year follow-up low CD4 counts were detected. [179]

Radiotherapy seems to prompt a temporary activation of the complement system; this was measured by C3a and C5a in both rodent and human tumors. In this case corticosteroids also seemed to suppress local activation of the complement system and thus diminish the positive effect of radiotherapy in relation to the immune system. [186] Specific components of the complement system also proved to play an integral part in the radiotherapy mediated tumor-specific immunity. When it comes to T-cell induction, C3aR1 and C5aR1 promoted differentiation into effector cells and the lack of this signaling tended to lead to a differentiation into regulatory T-cells. [187]

The abscopal effect

First described in the 50s where R.H Mole showed that irradiation of a tumor on one side also contributed to minimizing the untreated tumor located on the other side. [188]Abscopal is derived from the Latin word for "away from target". The theory

behind this event is that the irradiation generates an inflammatory response, providing tumor-specific antigens from the dying cancer cells directly affected by the radiation. It also provides maturation stimuli causing the dendritic cells to activate tumor-specific T-cells. [136]

Dewan et al could in their experimental mouse breast cancer model demonstrate the abscopal effect when irradiation was fractioned and combined with immunotherapy [183].

FLASH

FLASH-RT is characterized by an ultra-high dose rate (UHDR). Preclinical studies have shown that under certain conditions this type of dose delivery may increase the normal tissue tolerance compared to conventional (CONV) radiotherapy, without compromising the treatment effect. [189-192] In the majority of all treatment protocols and regimes spanning through all radiation modalities the standard dose rate has been a constant low one, around 0,1-0,2 Gy / s compared to UDHR that is characterized of dose rates at extremely high levels of > 40 Gy / s. [190, 193]

FLASH is not an entirely new invention, rather a rediscovered one. It was used by Dewey and Boag in the late 50s [194] and many others the following decades but seems to have been forgotten until Favaudon *et al*. published their study in 2014, where this approach to broaden the therapeutic window was proposed and by reducing the beam-on time from several minutes to a fraction of a second. Comparing FLASH vs CONV with a single dose of radiation delivered to the thorax of mice, a single dose of 17 Gy FLASH resulted in a dramatically lower degree of radiation-induced pulmonary fibrosis compared to 17 Gy CONV. [190] These results sparked a new interest for UHDR radiotherapy and its potentials. In continued studies a lower toxicity on normal tissue was confirmed in an variety of different animal models and organs[191], and also less dermal side-effects seen in a minipig and in cat and canine cancer patients. [193, 195] What remains, however, is to show the equally effective anti-tumor effect in vivo, which has not yet been fully explored, especially in an immunocompetent host. [190, 191, 196] Böhlen et al's did a literature search of publications evaluating in vivo tumor responses comparing FLASH-RT and CONV-RT. In this review from 2023, they found that the studies were predominantly based on limited numbers of animals and short follow-up. And most studies did not evaluate long-term tumor control. [197]

In the brain less inflammation was seen when FLASH-RT was compared with CONV-RT [198]. Better protection of blood vessels has also been noted. [199] In regard to neurotoxicity, several studies performed on mice models have shown spared neurocognitive functions. [196, 200, 201]

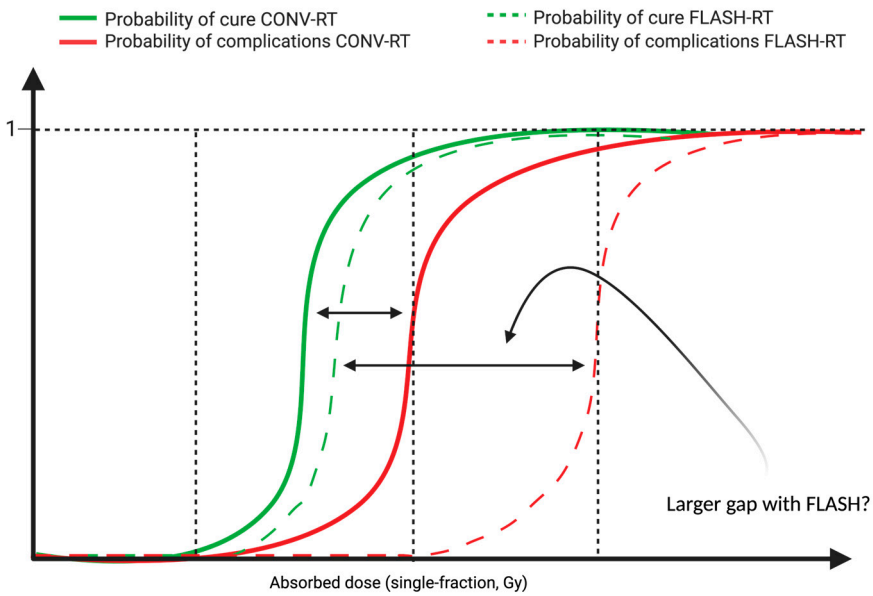
Previous studies in glioblastoma models have showed a postponed growth when studied in nude mice, while they seemed to develop less cognitive side effects when given FLASH compared to CONV-RT, as well as tumor growth delay being similar when comparing the two modalities. [196, 202]

The FLASH effect

Although the exact mechanism behind the FLASH effect is not fully known, there are a few established theories. It is to be noted that these protective effect seems to appear first at average dose rates greater than 30 Gy/s. [200]

The FLASH effect

Schematic overview of reasoning behind the FLASH-effect.
Created with BioRender.com



To thoroughly understand the reasoning behind the different theories we must once again discuss some of the basic mechanisms behind radiotherapy induced cell damage. These DNA damages are a combination of both direct and indirect effects. The indirect effect is the damaged generated by reactive oxygen species, ROS.

The direct damages caused through DNA damage, where double strand breaks are the most critical of DNA lesions and compromises genomic integrity. Cells have the ability to repair DNA through the DNA damage response pathway. This pathway is mediated by checkpoint kinases and have several outcomes depending on the damage type and severity. It can also decide to promote senescence or programmed cell death

depending on the damage repair outcome. These have become interesting targets for potential anti-tumor drugs, such as PARP inhibitors in BRCA1 or BRCA2, which are essentials in the genome stability. [203] Since cancer cells are more prone to have inadequately working repair-systems they are more at risk of not recover from an irradiation injury, whereas, healthy cells stand a better chance of surviving.

The mechanism behind the high levels of ROS in tumor cells compared to healthy cells, is not one but several underlying causes contribute. Mutations cause mitochondrial dysfunction which promotes metabolism, but mutation may also cause increased peroxisome activity, both of these leads to a higher production of ROS in the cell. [67] Tumor cells also often lack antioxidant enzymes in comparison to healthy cells, contributing to insufficient removal of ROS. [68]

It has been suggested that FLASH due to its ultra-high dose rate causes a transient hypoxia in the healthy tissue, which produces less reactive oxygen species and better removal of these. All together this results in overall less toxicity for the healthy tissue. In the tumor mass, on the other hand, there is already an adaption to the hypoxic environment and thus a slower removal of ROS which leads to cell damage that is comparable to the damaging effect CONV-RT has on the tumor tissue [204]. The reduction of ROS has also been shown to be beneficial in the case of cognitive decline [205].

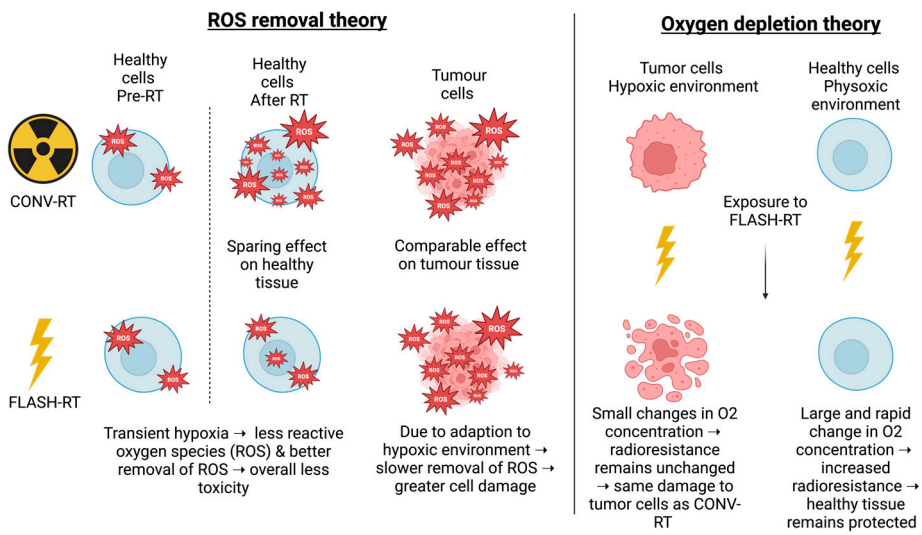
An additional hypothesis is related to the higher levels of iron in cancer cells. Iron is both key in enzymes involved in cellular respiration and DNA synthesis but also in the Fenton reaction. The Fenton reaction is a chemical reaction where iron and hydrogen peroxide react and produces free radicals, this reaction in itself has been suggested to cause mutations leading to cancer devolvement. [206, 207] Cancer cells are also known to alter iron metabolism. Partly by upregulating proteins such as transferrin receptor which increase iron uptake, which is common in glioblastomas and gliomas and thus also is potential target for treatments. [208, 209] Published earlier this year was Marrocco et al's innovative study where they utilized ferritin-based nanovectors administered intranasally in a glioblastoma mouse model and showed improved survival rates. [210] The overall higher levels of iron present in the tumor cells make them more susceptible to further oxidative damages when exposed to FLASH induced hydroperoxide. Healthy cells are able to remove hydroperoxide more swiftly due to their lower levels of iron. [211]

Several authors have investigated the role of oxygen in the FLASH-effect. In the late 60s and early 70s Town et al as well as Epp et al explored this. Town et al found a higher level of sparing non-cancerous mammalian cells. [212] Epp et al studied HeLa cells and FLASH in different oxygen concentrations and found the same results of higher levels of survival in hypoxic conditions. [213] In more recent years our colleagues, co-authors and co-supervisor here in Lund have explored the same theory. Adrian et al compared FLASH-RT to CONV-RT in both normoxic and hypoxic environment on a prostate cancer cell line, DU-145. The evaluation with

clonogenic assay showed no difference in survival between the two modalities in normoxic conditions, however, under hypoxic conditions there seemed to be a sparing effect on the cancer cells that received FLASH-RT. This was seen mainly at higher doses of 15 and 18 Gy. [204] This also highlights the importance of further studies of FLASH-RT to reassure the efficacy in vivo. In another study, seven human cancer cells lines and one normal lung fibroblast cell line was compared exploring lower doses of 0 to 12 Gy. An increased survival was suggested in all cell lines, suggesting a sparing effect of FLASH even in lower doses under normoxic conditions. The extent of which the FLASH-effect was seen differed between the cell lines, leading to the conclusion that the FLASH-effect is not only explained by the theory of transient oxygen depletion. [214] In regard to this topic it is important to mention that physiological oxygen level differ for every healthy tissue and then additionally differ when it comes to tumors. [215]

Foulliade et al examined the effects on normal pulmonary cells as well as stem cells and the radio-induced DNA-damage. They found that FLASH overall contributed to less DNA damage in normal cells but also that FLASH spared stem cells to a greater extent. The latter of which is of importance in maintaining stemness and recovery. [216]

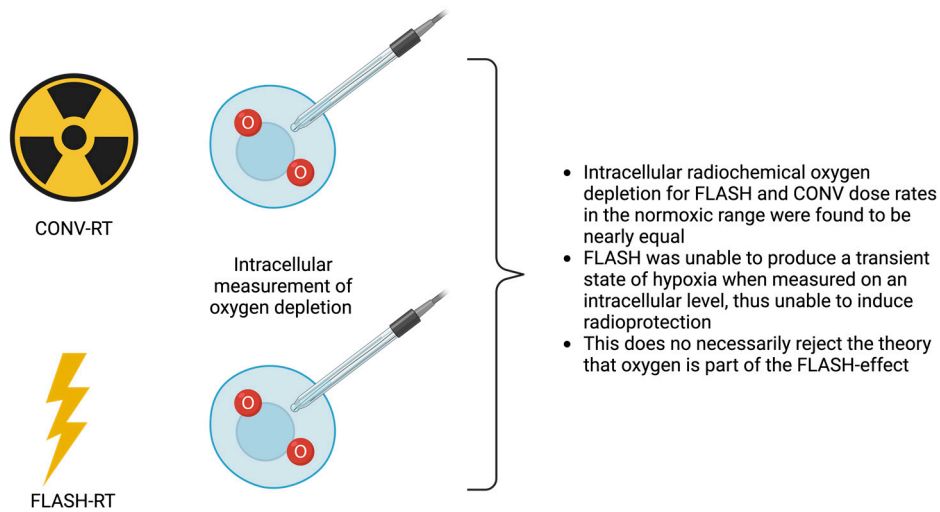
Schematic simplified overview of theories behind the FLASH-effect
Adapted from figures by Montay-Gruel et al's "*Long-term neurocognitive benefits of FLASH radiotherapy driven by reduced reactive oxygen species*" and Wilson et al's "*Ultra-High Dose Rate (FLASH) Radiotherapy: Silver Bullet or Fool's Gold?*".
Created with BioRender.com



However, most studies that have been performed have measured oxygen levels in either the interstitial space or blood plasma. El Khatib et al published their study where they measured intracellular oxygen depletion by using electroporation. Here

they found no transient hypoxia in the cytosol after a single dose of FLASH-RT. There was no difference between radiolytic oxygen depletion within the cells when comparing CONV-RT to FLASH-RT. The authors, stated however, that this does not mean that the theory behind the FLASH-effect being oxygen dependent should be discarded but rather it gives us an additional insight into the mechanism. [217] Another interesting theory behind the FLASH effect is its effect on the immune system, mainly surrounding lymphocytes and its supposedly sparing effect. It is suggested that due to its shorter time fewer lymphocytes are irradiated, thus leading to less lymphopenia, a known side-effect of radiotherapy. [180, 218, 219] Jin et al, Cucinotta and Smirnova as well as Galts and Hammi used mathematical models or simulations to evaluate the effect on circulating lymphocytes. In both cases FLASH therapy spared circulating immune cells compared to CONV-RT. [218-220] More of interest to this thesis is the findings of Galts and Hammi's as their model was based on intracranial treatment, although proton based, they saw an improved result when FLASH was administered as a single dose fraction during intracranial treatment. [219]

To conclude, transient hypoxia has been a leading theory behind the FLASH effect, but in time it has become apparent that it is not the sole source behind this elusive phenomenon.



Schematic simplified summary of results presented by El Khatib et al
From their publication "*Direct Measurements of FLASH-Induced Changes in Intracellular Oxygenation*", published in 2023.
Created with BioRender.com

Continuing with results especially interesting in the field of GBM research are the promising results of Montay-Gruel et al using hypo-fractionated FLASH-RT in a GBM mice model, showed sparing effects on the normal brain when comparing FLASH to CONV in novel object recognition, without seeming to compromise the anti-tumor effect. [196] This was published following their first study in 2019 that showed long-term neurocognitive benefits. In this extensive study they evaluated neurocognition using several tests for the mice as well as markers of minimized neuroinflammation. Neuroinflammation was studied in depth by examining GFAP+ cells, where FLASH indicated a lesser amount of reactive gliosis. FLASH-RT also seemed to prevent increased microglial activation, when comparing Cd68+ expression. Lastly, they also examined neuronal morphology by looking at dendritic area, spines, branches and length. Both at two months post radiotherapy as well as at the 6-month follow-up, rodents irradiated with FLASH-RT showed preserved structures compared to CONV-RT. [205]

Overall, these findings of healthy tissue sparing contributes to a larger dose range in FLASH compared to CONV. But one of the fears with FLASH-RT has been that due to its tissue sparing effects it would also spare the tumor cells. However, in recent studies FLASH-RT has been shown to be equally effective as CONV-RT in anti-tumor effect. [190, 196, 202, 221-224]

However, it is extremely important to secure the anti-tumoral effect of FLASH in different models, including long-term tumor control and fully immunocompetent models.

Chemotherapy

Temozolomide

Is an alkylating agent with antitumor activity, both as a single treatment but also as part of the Stupp protocol. TMZ affects single strands of DNA and preferentially methylates DNA at specific sites. [10] One of its many benefits is its lipophilic qualities, therefore able to cross the blood-brain barrier and can be administered orally to the patients. [225] As mentioned previously, the MGMT-gene, which encodes a DNA-repair protein, is one of the few known prognostic markers for GBM. TMZ alkylates the O6 site on guanine, which leads to the formation of O6-methylguannine adducts, which promotes single- and double-stranded DNA breaks resulting in cell cycle arrest and apoptosis. The DNA-repair protein coded by the MGMT gene can remove the alkyl groups from the O6 position of guanine, therefore high levels of MGMT protein in cancer creates a resistance to alkylating agents. [226] Even though TMZ is a vital part of the GBM treatment it is also part of tumor resistance and recurrence, where TMZ resistance unfortunately is common. I have previously discussed the implications of GBM heterogeneity, specifically intratumoral heterogeneity and its role and response to treatment. When discussion

TMZ resistance, or antitumoral treatment resistance overall, there are two types, intrinsic and acquired. The ongoing large research into the field has identified several suspected underlaying mechanisms that combined produces TMZ resistance in GBM. [227]

Nitrosoureas

Nitrosoureas include DNA alkylating agents such as Lomustine. They are beneficial in their ability to cross the blood–brain barrier, due to their high lipophilicity.
After several Phase II trials TMZ is still the first line chemotherapy even for recurring GBM. Lomustine or CCNU (chloroethyl-cyclohexyl-nitrosourea) is a chemotherapy drug used in recurrent GBM. It is an alkylation agent and one of its most prominent lesions can be reverted by MGMT. It is a lipid-soluble drug and may therefore penetrate the BBB. Overall, clinical trials of recurrent GBM with Lomustine as a control arm, showed a low response rate and short progression free survival. [228-235] Few of these trials have reported MGMT promoter methylation status, and among those that did, low or no activity at all was seen in patients lacking this methylation. [231, 233, 234] It is quite apparent that there is a lack of solid scientific studies in the evaluation of Lomustine in the recurrent GBM regime. In Weller and Le Rhun's review [236] they point to the small study by J. Duerinck et al from 2017, comparing Axitinib, a small tyrosine kinase inhibitor, alone to its combination with Lomustine, showing no additional benefit of Lomustine. [232] So far, the best data to rely on is the one coming from the PCV protocol, which is Lomustine in combination with one more alkylating agent Procarbazine and an antimitotic agent Vincristine. First evaluated in 1975, proving to be a superior method to Carmustine, [237, 238] the PCV regime has been shown to be beneficial when combined with RT compared to RT alone in randomized clinical trials on lower grade gliomas. [239-242] Its efficacy in high grade gliomas has not been confirmed in a clinical trial. Regarding the PCV regime, Vincristine does not cross the BBB, making its role in the regime questionable and suggestions has been made to remove, also due to its side effects. [236]

Carmustine (BCNU) is also an alkylating agent, that originally was administered intravenously but more recently also in the form of wafers, known as Gliadel wafers, administered locally during surgery. The Gliadel wafer was developed by Brem et al in the 90s to avoid systemic treatment and its associated side-effects. [243-245] Roux et al showed it to at least provide a therapeutic bridge following the pause of treatment from surgery awaiting start of radiotherapy. [246] Although being an innovative way to combat GBM it did not lead to any cure and is not often used in today's praxis.

Other treatments

Optune or Tumor-treating fields

Optune was introduced in 2009 in a clinical trial by Stupp et al leading up to their publication in 2017 of *"Effect of Tumor-Treating Fields Plus Maintenance Temozolomide vs Maintenance Temozolomide Alone on Survival in Patients With Glioblastoma - A Randomized Clinical Trial"*, which showed improved progression-free survival as well as overall survival. Patients treated with TMZ + TTF had an overall median survival of 20,9 months, compared to 16,0 months for the group that received TMZ alone. [247] It is now a well-established additional treatment in combination with Temozolomide. It is hypothesised that the electric fields interfere with cell division, by exerting forces on the charged particles within the cells. More specifically it is believed to affect the spindle formation during the chromosome separation during the division, causing cell cycle arrest and ultimately cell death. Cancer cells are more likely to be affected due to their much higher rate of division compared to normal healthy tissue. [248-250] The patient is equipped with several electrodes to the scalp. So far, few adverse effects have been noted, mostly just local skin reaction, but the treatment as such can be quite demanding for the patient since the equipment has to be in place for many hours every day. [247]

Bevacizumab

Bevacizumab is a monoclonal antibody, it inhibits the effects of vascular endothelial growth factor, VEGF-A, known to be upregulated in glioblastoma and contributing to its characteristic angiogenesis. [251, 252] Bevacizumab has since the early 2000s been approved as a treatment for several cancer types in the United States and was approved for recurrent glioblastoma in adults in 2009. [253, 254] However, the efficacy of this treatment in terms of anti-tumoral effects is very limited. In 2014 Gilbert et al as well as Chinot et al, performed randomized control trials that showed no additional overall survival for patients receiving Bevacizumab added to the radiotherapy-TMZ regime. [255, 256] It is sometimes used as a corticosteroid sparing alternative to reduce peritumoral edema. [257]

IDH-inhibitors

In 2023 the results from the INDIGO trial, a global and randomized, double-blinded, phase 3 study of Vorasidenib, an IDH-inhibitor, was published by K. Mellinghoff et al. Vorasidenib or placebo was given to patients with residual or recurrent grade 2 glioma with an IDH1/2 mutation and primary end point was imaging-based progression-free survival. Progression free survival was significantly improved in the group receiving Vorasidenib with a median of 27,7 months compared to 11,1

months in the placebo group. Secondary end point was the time to the next anticancer intervention, which once again was improved in the Vorasidenib group. It is also of interest to note that IDH-mutation have been found in other tumors such as acute myeloid leukaemia and chondrosarcoma, where potential benefits from IDH-inhibitors might also be found. [258]

CAR-T

Chimeric antigen receptor (CAR) T cells, first approved by the FDA in 2017 and by EMA in 2018, are programmed receptors targeting tumor-specific antigens. [259, 260]

First to be approved were therapies targeting CD19 on B-cells, thus targeting B-cells malignancies. However, during the las 6 years since its first approval there is now an array of different targets being evaluated. For glioma and glioblastoma mainly the following: [261]

- Epidermal growth factor receptor (EGFR)
- Human epidermal growth factor receptor 2 (HER2)
- Interleukin-13Ra2
- Cluster of differentiation 133 (CD 133)
- Erythropoietin-producing human hepatocellular carcinoma A2 (EphA2) [262]
- GD2 disialoganglioside [263]

Although in initial studies proven safe with little toxicity, the issue of glioblastoma heterogeneity remains. With great excitement however, interim results were published from an ongoing phase 1 trail during 2024. In this protocol bivalent CAR-T cells, targeting EGFR and IL13Rα2, have shown promising results. [264] A major issue to take into consideration however, is the results shown by O'Rourke et al, with development of adaptive resistance and antigen loss after only one dose of EGFRvIII-directed CAR T-cells in glioblastoma patients, once again showing the illusive and illicit manner of glioblastoma and its reoccurring challenges. [265] As known from other malignancies, CAR-T therapy is not without significant risks for the patients and sometimes lethal complications, including severe brain edema, known as immune effector cell-associated neurotoxicity syndrome or ICANS. [266]

Rationale

Hopefully by now it has become apparent to the reader that despite the immense research put into this dreadful diagnosis the expected survival, with all available treatment options, is still only 12-15 months. The explanation lays in the illusive nature of glioblastoma, its inherent nature to somehow always find a way to outsmart us.

Many studies performed have not included a fully immunocompetent animal model and therefore lacks the important interplay between glioblastoma and the immune system which further contributes to its complexity.

There is a similar lack of studies on the rediscovered FLASH radiotherapy and its prospects of being sparing on normal tissue while equally as effective as conventional radiotherapy for tumor control.

This combined presented a current void in the area in which these studies hope to bring some additional understanding.

Aims

Paper I

I. If there was a difference in anti-tumor efficacy following radiotherapy with CONV-RT or FLASH in fully immunocompetent animals irradiated with low total doses in an early phase of tumor growth, with 8 Gy × 2 for subcutaneous tumors, and 12.5 Gy × 2 for intracranial tumors.
II. If irradiation with CONV-RT or FLASH could generate long-term antitumor effects in fully immunocompetent animals.
III. The effect of the local tumor microenvironment, comparing effects in subcutaneous tumors to those seen in the intracranial setting.

Paper II

IV. Evaluate and compare tumor control and treatment toxicity for various doses of hypofractionated FLASH-RT versus CONV-RT by assessing overall survival, tumor growth, and long-term tumor control.
V. The frequency of acute and late local dermal toxic effects comparing FLASH-RT versus CONV-RT.

Paper III

VI. Does single-fraction irradiation with FLASH-RT and CONV-RT have equal anti-tumor efficacy against intracranial glioblastoma?
VII. Does tumor size differ between animals depending on irradiation dose?
VIII. Are there any differences between FLASH-RT and CONV-RT when it comes to dermal side effects?

Paper IV

IX. Exploring the efficacy of combined radiotherapy and anti-C1-INH antibody treatment in experimental glioblastoma.
X. Comparing efficacy in intracranial versus subcutaneous tumor microenvironments.

Ethics

Ethical statements for each paper

Paper I

This study was approved by the Animal Ethics Committee in Malmö/Lund, Sweden (permit ID 5.8.18-02383/2020 and 5.8.18-04987/2021). All experiments were performed in accordance with relevant guidelines and regulations. All efforts were made to minimize animal suffering and in accordance to ARRIVE guidelines.

Paper II

This study was approved by the animal ethics committee at Lund University with permit ID 5-8-18-02383/2020 and amendment 2021. All efforts were made to minimize animal suffering.

Paper III

The study was approved by the Regional Ethics Board in Lund-Malmö, Sweden (ethical permission number 1469-2022).

Paper IV

This study was approved by the Malmö-Lund Animal Research Ethics Committee (permit ID 5–8-18–02,383/2020). All experiments were performed in accordance with relevant guidelines and regulations. All efforts were made to minimize animal suffering and in accordance with ARRIVE guidelines.

Ethical self-reflection

Glioblastoma is our most common primary brain tumor among adults and with a poor prognosis, comparable to pancreatic cancer. The limited treatment options today consist of; surgery followed by chemotherapy and radiation, but still there is no cure. Our research focuses on possible new treatment options for this detrimental disease.

Since our research does include animal testing, I think this is the main ethical issue worth discussing.

I personally have found it very challenging on an ethical level when I started conducting trials on our rats. The animals have of course never approved this, and they will never perceive any benefits of the research. I very much understand the criticism and urge to ban animal testing but, I think that there are several misconceptions regarding animal testing in medical research that I would like to address. For example, I think that a great majority is under the impression that it is an option to test new experimental treatments on humans directly, which of course is not the case. Especially when we are early in our research and still lacking fundamental scientific knowledge, this would be highly unethical.

We not only try to use alternative methods to test our hypotheses as often as possible but are obligated to do so, however, it is not always an option. I think that few who object to animal testing in medical research consider the patients of said disease and their need for continuous research and subsequently also animal testing to give them hope of not only cure but also prolonged life and improved quality of life. Perhaps it is easy to condemn this research when it seems far away, but what if this disease affected one of your loved ones?

In regards of reduction, we always make power calculations and use the least number of animals needed to still be able to get significant results because it is a careful evaluation because you of course want to use as few animals as possible. But you also want to make sure that you have enough animals to achieve significant results, otherwise the entire trial is a waste, and the suffering of the animals included would be in vain. We try to start out with pilot trials to confirm that our model is working and only then move on to greater quantities. Recently we evaluated different doses of radiotherapy on our cell-line instead of directly on the animals and thereby also minimized the number of animals used because we already had a preliminary notion of what doses to use because of our trial on the cell-line. However, the best way to fully evaluate the immune response is by using a fully immunocompetent animal-model.

When it comes to refinement, our rats are put under anaesthesia using gas inhalation and for transportation, they are given an injection with additional anaesthesia medication for prolonged effect. As they are already asleep, they do not feel any pain of the additional injection.

In conclusion, taking into consideration that our research aim is to find a possible new treatment and that our research is still in its early stages, the only possibility to give this group of patients any hope is to continue our research and when necessary, by utilizing animal models. Even if we always aim to use alternative models, it is simply not possible for us to fully evaluate the efficacy in vivo as well as the immune response.

Methods

NS1 cell-line

Many of the glioblastoma cell lines used both in vitro and in vivo have been processed for a long time and due to this lost many of its characteristics along the way. This, in addition with being able to produce a GFP positive tumor line that could be used in a fully immunocompetent animals and an easily tracked glioma model, were the starting points of the making of the NS1 cell-line. NS1 is a GFP (green fluorescent protein) positive tumor cell line. Rats inoculated with NS1 cells develop cell-rich tumors with an invasive growth pattern. Tumors are positive for glial fibrillary acidic protein, GFP, and express wild-type isocitrate dehydrogenase 1 (IDH-wt). [267]

Development

Pregnant GFP positive female rats, with homozygous GFP positivity, were treated with 60 mg/kg of ENU i.v. at estimated gestation day 15. Later the offspring was followed for any clinical signs of tumor growth. When noticed the animals were euthanatized and the brain and medulla were dissected. Tumor cells were then transplanted into new recipients, until a stable glioblastoma cell line was established. [268]

Characteristics

Later PCR analysis was preformed, expressions shown included IDO1, PDL1 and EGFR. High levels of wild-type IDH1and wild type un-mutated p53 were identified. The expression was then also tested after radiation. [267]

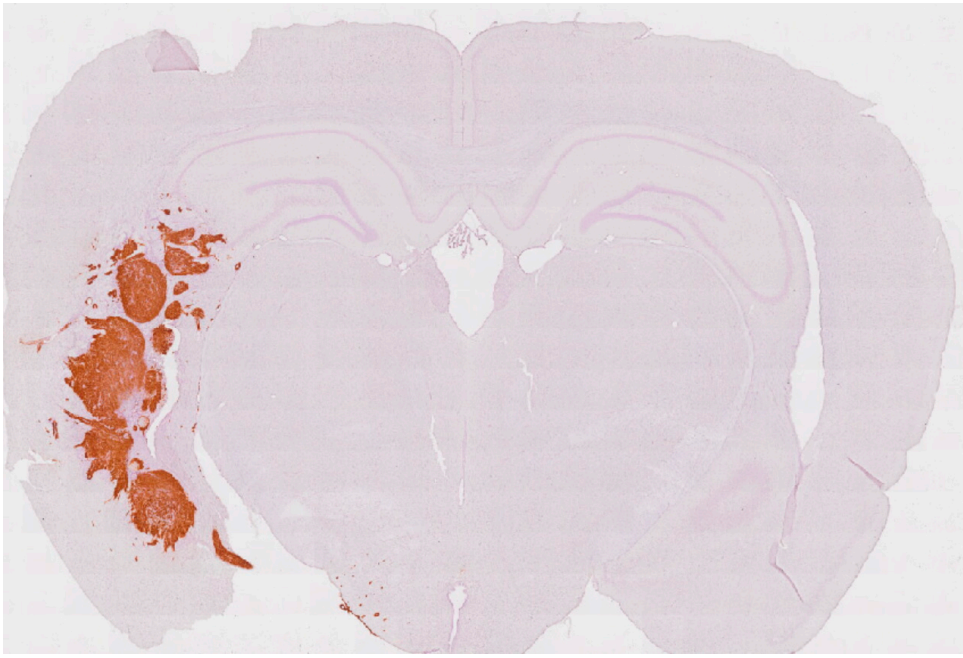


Image with rodent brain stained with both anti-GFP antibodies and htx-eosing, shows the infiltrating tumor cells of NS1. This rodent was irradiated with a high dose at the site of inoculation (this animal was not included in the studies of this thesis). This image shows that the tumor has progressed otuside the field of irradiation.

Histopathology of NS1

With htx-eosin staining cell rich tumors could be identified both i.c. and s.c., with a characteristic central necrosis and pseudopallisading. Transplanted tumor cells into a GFP-negative Fischer 344 rat infiltrating GFP positive cells could be identified both intracranially and subcutaneously.

Preparation of cells

Sandwich Elisa was used to rule out Mycoplasma infection in the cells and supernatant and was used according to the manufacturer's instructions (MycoProbe R&D Systems).

NS1 cells were prepared for inoculation as previously described by us [269]. In brief, cells were seeded in IMDM culture media with 10% fetal bovine serum, 1% sodium-pyruvate (100mM), 1% Glutamax (100X) and 0.05 mg/ml gentamycin (all from Gibco) and cultured in 37°C and 5% CO2 in a humidified incubator. Trypsin (Invitrogen) was added, and cells were incubated to detach the adherent cells. Cells

were centrifuged at 1200 rpm for 5 minutes at 4°C, and then the supernatant was removed. Afterwards the cell pellet was re-suspended in serum-free medium.

In vivo experiments

Fully immunocompetent Fischer 344 rats were used (Fischer Scientific, Germany) as previously described by us [137, 269]. The rats were housed in pairs in rat cages with water and rat chow *ad libitum.*

All efforts were made to minimize animal suffering.

Intracranial inoculations

In the intracranial model, each rat was inoculated with 5000 cells from the NS1cell line, suspended in 5 µl of nutrient solution. The procedure was done under isoflurane inhalation anaesthesia using a stereotactic frame. Schematic image of the stereotactic frame used is shown in the images under experimental set-up. To inject the cells a 10 µl Hamilton syringe was used. In all studies the cells were injected on the right side of the cranium at a depth of 5 mm, 2 mm laterally from the sagittal suture, and 1 mm anterior to the coronal suture. The cranial burr hole was sealed with bone wax, and the incision was closed with absorbable suture.

The animals were monitored daily, and those displaying signs of paresis, epilepsy, or declined general condition, were euthanized with CO2 inhalation according to the ethical permission.

Subcutaneous inoculations

To establish subcutaneous tumors, rats were inoculated with 50,000 NS1 cells subcutaneously day 0 in prone position in the hindleg. The tumor site was carefully marked. The inoculation was performed during general anesthesia with isoflurane inhalation. Animals were monitored daily, and those showing signs of tumor diameter >30 mm or declined general condition were euthanized. The criteria for euthanasia are defined in the animal ethics permission and in accordance with the acceptance from the ethics board.

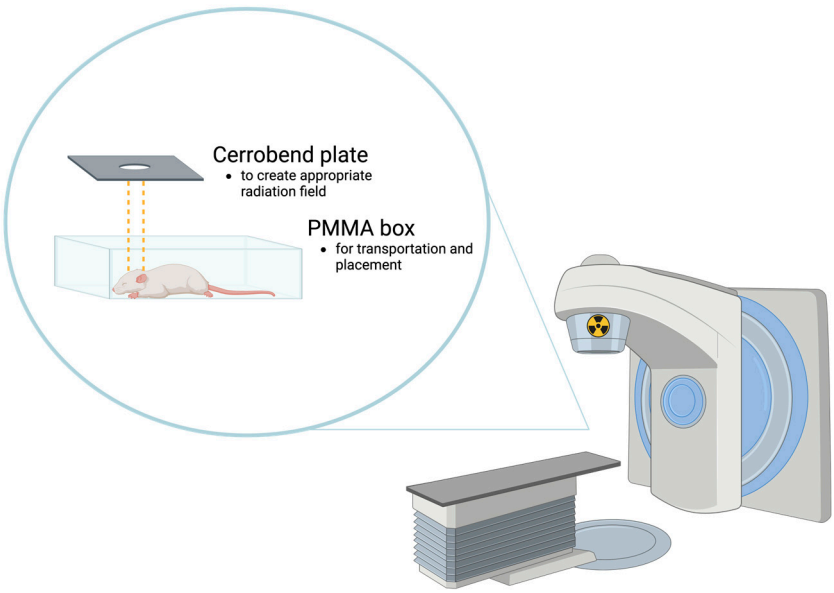
In Paper I, animals who survived 91 days with no signs of tumor growth were re-challenged, with a tumor inoculation with NS1 cells on the contralateral side compared to the primary inoculation. The animals were followed until day 191 from the first inoculation and were monitored regarding signs of tumor re-growth on either side.

Radiotherapy

Prior to irradiation, the animals were anaesthetized by intraperitoneal injection of Ketalar/Rompun and positioned in custom-made PMMA boxes. Tumors were targeted using the crosshair of the linear accelerator light field (with the inoculation site as a reference point for intracranial tumors).

The animals were irradiated using a 10 MeV electron beam of a clinical linear accelerator (Elekta Precise, Stockholm, Sweden). For FLASH-RT delivery, the treatment machine was temporarily modified to deliver ultrahigh-dose-rate electrons in 3.5 µs pulses, as described elsewhere. [270]

The dose-per-pulse (for FLASH-RT) and dose-per-monitor-unit (for CONV-RT) in this position was determined using GafChromic film (XD film, Ashland Advances Materials, Bridgewater NJ) at 5 mm depth in a polysterene phantom placed in one of the boxes. Film measurements were repeated prior to each treatment session to verify the delivered dose. During administration of FLASH-RT, a Farmer-type ionization chamber was used for relative output measurements to ensure output stability in FLASH-RT mode.



Experimental set-up for radiotherapy
Set up and modifications at the clinical linear accelerator during rodent radiotherapy. The cerrobend plate was moved and placed accordingly depending on tumor location.
Created with BioRender.com

The doses of irradiation were chosen based their ability to elicit an immune response, if not by itself then in combination with immunotherapy [137, 183, 271]. Doses were increased for the intracranial tumors based on prior findings from our research on this tumor model [137].

In vitro colony forming assay

In vitro survival of irradiated cells was evaluated using colony forming assay. Cells were plated in T12.5 flasks (Thermo-Fisher Scientific, Waltham, MA) in a total volume of 2.5 ml, and allowed to adhere overnight. Flasks were irradiated in triplicates with various doses, either with CONV-RT (8 Gy/min) or FLASH (average dose rate > 90 Gy/s, 3 Gy/pulse). After irradiation, flasks were incubated in 37 °C for 7 days for colonies to form, and then stained using methylene-blue in ethanol for evaluation. Unirradiated control flasks were used to determine plating efficiency. Colonies containing at least 50 cells were defined as survivors.

Intracranial tumors

Animals were irradiated using a field size of $1 \times 1 cm^2$. CONV-RT was delivered with an average dose rate of 8 Gy/min. For FLASH, each fraction was delivered in pulses varying from 7-15 pulses depending on the dose given. Each with an average dose rate of 74 Gy/s up to >459 Gy/s for the higher doses, with a total treatment time of up to 170 ms.

The absorbed dose was prescribed at 5 mm depth, i.e., at the same depth as the tumor cells were injected.

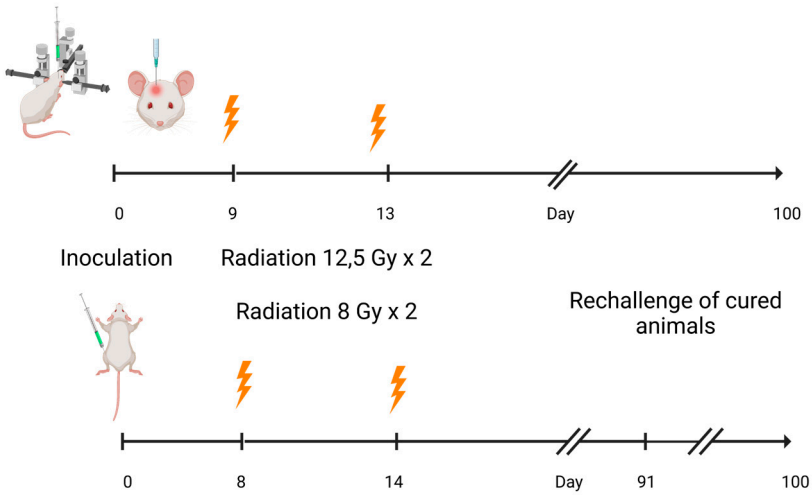
Subcutaneous tumors

Animals were irradiated with a circular radiation field with a diameter of 2 cm. CONV-RT was delivered at a source-to-surface distance (SSD) of 65 cm with an average dose rate of 8 Gy/min. For FLASH, each fraction was once again delivered at SSD = 65 cm in a number of pulses, varying from 4 to 8, depending on dose. With an average dose rate of 66-70 Gy/s, and a total treatment time of ≤180. Dosimetry was performed using GafChromic XD film (Ashland Advanced Materials, Bridgewater NJ) prior to each treatment. For FLASH delivery, a Farmer-type ionization chamber (NE 2505/3-3A) was used for relative output measurements during treatment.

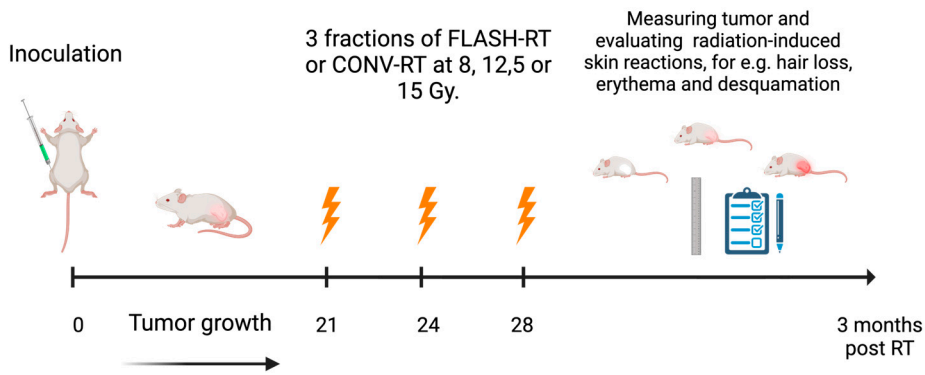
For both CONV-RT and FLASH-RT irradiation, an electron applicator was fitted with a Cerrobend plate creating a circular radiation field of 3 cm in diameter.

The absorbed dose was prescribed at 4-mm depth in the animal. A custom-made holder was used for relative output measurements to ensure output stability in FLASH-RT mode.

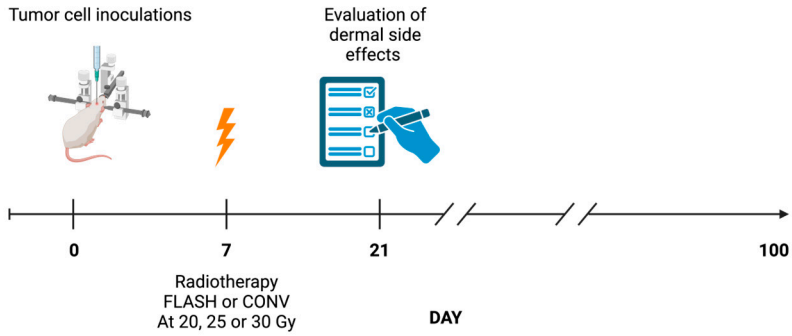
Overview of experimental set-up in each paper



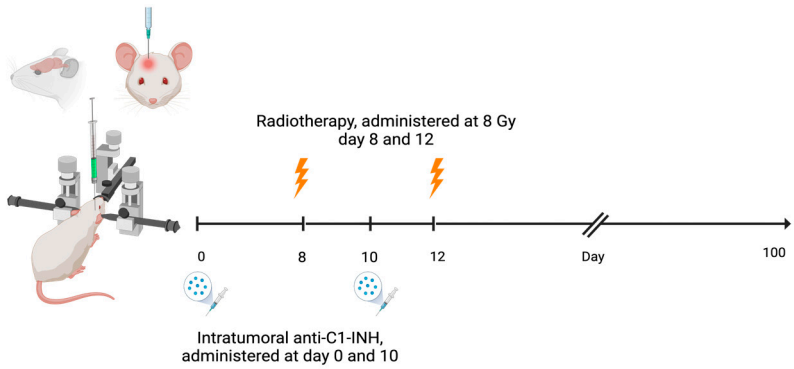
Paper I. Animals were divided into 3 groups receiving FLASH, CONV or non-irradiated controls. At day 91, 14 animals in the subcutaneous group did not exhibit any sign of tumor growth and were inoculated de novo with NS1 on the contralateral flank. *Image created with Biorender.*

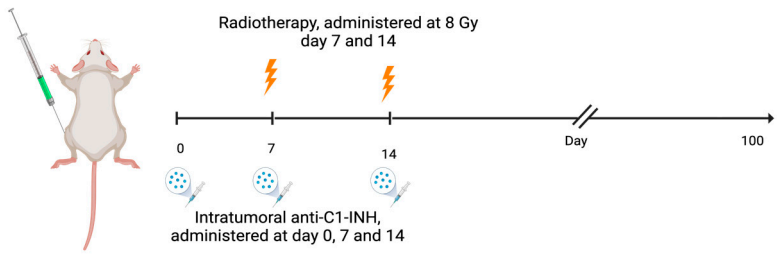


Paper II. Animals were randomly assigned between groups according to tumor size on day 18 after inoculation. Animals were inoculated in prone position on day 0 and irradiated with either CONV-RT or FLASH-RT on day 21, 24, and 28. *Image created with Biorender.*



Paper III. Radiotherapy was administered at day 7 as a single fraction of either 20, 25, or 30 Gy, using CONV-RT or FLASH-RT. Dermal side effects were then evaluated at 14 days after irradiation. *Image created with Biorender.*



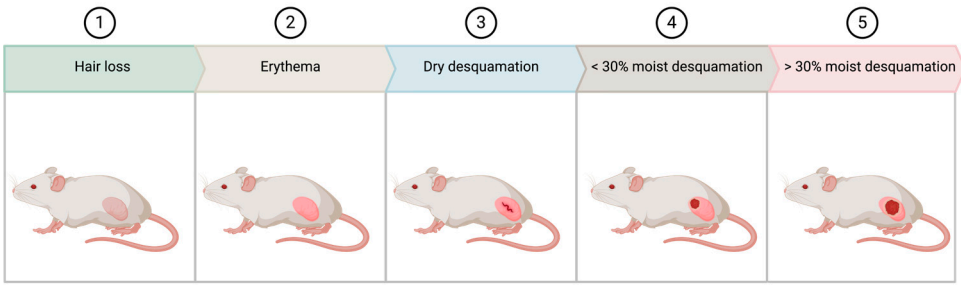


Paper IV. Animals with intracranial tumors were injected intratumorally with 0.4 µl anti-C1-INH (6.15 mg/ml) (Covance) on days 0 and 10 using the same stereotactic and the same burr hole and at the same depth. Animals with subcutaneous tumors were treated with 0.1 ml anti-C1-INH (6.15 mg/ml) (Covance), administered by intratumoral injections on days 0, 7 and 14. *Image created with Biorender.*

Dermal side-effects

Animals were evaluated for acute radiation-induced skin reactions according to a phenotypic grading scale of 1 to 6 (1: normal, 2: hair loss, 3: erythema, 4: dry desquamation, 5: <30% moist desquamation, and 6: >30% moist desquamation) established by de Andrade et al. [272]

Observations and toxicity evaluations were performed weekly, 2 to 5 weeks after completed RT. Animals that were euthanized due to large tumors (diameter >30 mm) during the study period were excluded from the analysis to avoid mistaking a sub-therapeutically treated fast-growing tumor for local side effects to the skin. At 3 months after initiation of RT, the surviving animals were evaluated for late skin toxicity by determining the ratio of animals with toxic effects greater than grade 1.



Skin reactions ranking
Based on images from de Andrade et al's publication "*Radiotherapy-Induced Skin Reactions Induce Fibrosis Mediated by TGF-β1 Cytokine*" from 2017.
Created with BioRender.com

Histopathology

After euthanasia, brains were carefully removed and fixed in 4% formaldehyde followed by paraffin embedding and microtome sectioning of the samples to 7µm thickness.

Brain samples were stained using a primary biotinylated goat anti-GFP antibody (ABIN1000087, antibodies-online), a VECTASTAIN® ABC-HRP goat IgG kit (PK-4005, Vector Laboratories), and a DAB detection kit (Agilent Dako). Mayers HTX (HistoLab) was used as a counterstain.

Samples were mounted on microscope slides using Pertex® mounting media (HistoLab).

Tumor size was analyzed with light microscopy, blinded to the treatment situation, and graded accordingly. The brains were investigated both frontally, at the site of the coronal suture, and posteriorly.

Immunohistochemistry

Antigen identification was performed on de-paraffinized sections. Sections were submerged in pre-heated (100 °C) citrate buffer (Citrate Buffer, pH 6.0, 10 ×, Antigen Retriever, Sigma-Aldrich) for 20 min and washed with PBS before immunohistochemistry.

Detection of CD8, CD4 and FOXP3 was performed using ready-to-use Vectastain ABC kit (Vector Laboratories, CA, USA) in combination with primary antibodies consisting of rabbit anti-CD8 (1:200, Sigma-Aldrich, MSA48-GA), mouse anti-CD4 (1:200, Sigma-Aldrich, SAB4700733), and rabbit anti-FoxP3 (1:200, Antibodies online, ABIN3187942), individually.

Images were captured using an Olympus VS120-26–096 Virtual Slide Microscope with a × 20 objective using and Olympus VS-ASW 2.9 software.

Multiplex Assay in Paper I

Luminex Multiplex Assay (Bio-Techne) was used on rat serum according to the manufacturer's instructions, including the analytes GM-CSF, ICAM-1, IL-2, IL-6, IL-18, TIMP-1, TNF-alpha and VEGF.

Gene analysis

In Paper I and Paper IV part of the tumor was dissected and frozen in liquid nitrogen when the animals fulfilled criteria for euthanasia. RNA extraction was performed using RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA sequencing was performed at Center for Translational Genetics, Lund University. Only samples with adequate RNA quality could be assessed. Data was analyzed in R v.3.6.3 (R core team 2020) [273-275]. All genes where two or more samples had fewer than 10 reads were removed prior to the analyses. The raw read counts were normalized to reads per million reads (rpmr) for each sample. Individual genes were considered expressed different, if there were at least a twofold change between groups of samples and the fold change was significant ($p < 0.05$ after correction for multiple testing) and adjusting for expression levels using a generalized linear model provided in the R package as previously described [269].

Tumor growth, overall survival, and treatment response

In the subcutaneous model the maximum tumor diameter was measured for all animals once every week. Measurements were carried out by the same personnel throughout the study, blinded to the assigned treatment group.

Animals were monitored daily, and overall survival was determined as the number of days from inoculation (day 0) until the criteria for euthanasia were reached.

Statistics

SPSS was used for statistical evaluations, except for gene analysis, where R was used. Normality was assessed using Shapiro–Wilk's test and visual inspection of normality plots. Kruskal–Wallis and Mann–Whitney U-tests were performed for non-parametric data, and Bonferroni corrections were used in cases of multiple hypothesis testing. ANOVA test was performed on parametric data, and Bonferroni corrections were applied in cases of multiple hypotheses testing. Fisher's exact two-sided test was used for comparison between the groups regarding tumor size. Spearman's two-sided test was used for evaluation of correlation.

Results

Paper I

Long-term anti-tumor effects following both conventional radiotherapy and FLASH in fully immunocompetent animals with glioblastoma

The aim was to explore whether a long-lasting anti-tumor response could be achieved using CONV-RT or FLASH in our animal model. Especially since the then current studies on FLASH-RT lacked data from a fully immunocompetent rat glioblastoma model. In addition, we explored irradiation on not only intracranial tumors, but also subcutaneous. The advantage of studying rat models is the consideration of them to be physiologically more similar to humans compared to other rodents such as mice [276]. It is also worth noting that previous studies often were performed on non-immunocompetent models, which made it hard to explore long-term anti-tumor effects [196].

In vitro data

The colony forming assay showed that the survival depended on both dose level and dose rate. At 18 Gy, we saw a significantly increased survival in cells irradiated with FLASH, as compared to CONV-RT (Mann–Whitney U-test, $p < 0.05$). At a dose of 21 Gy, no colonies with ≥ 50 cells were detected.

Significant anti-tumor effect of both FLASH and CONV-RT versus control in fully immunocompetent animals with subcutaneous glioblastoma

Survival was significantly increased in the irradiated animals compared to control animals. There was however no significant difference in survival between FLASH versus CONV-RT.

Tumor size differed significantly between control animals and those treated with FLASH 8 Gy × 2 or CONV-RT 8 Gy × 2, at measurements 14–49 days after tumor cell inoculations. Significant differences were observed already after 14 days. Differences in tumor size remained throughout the observation period, as seen after 20 days and also at 49 days, the last day when all animals were still alive.

At 49 days, the first control animals had to be euthanized due to large tumors. Still no significant difference between animals irradiated with CONV-RT or FLASH was seen at any of the time points between day 0 to 49 after tumor cell inoculations.

Long-term anti-tumor effects in cured animals with subcutaneous glioblastoma

91 days after inoculation, 14 animals did not exhibit any sign of tumor. 8 treated with FLASH 8 Gy × 2 and 6 with CONV-RT 8 Gy × 2. All control animals had been euthanatized at this point due to tumor diameter > 30 mm. The animals with no signs of tumor were inoculated with 50,000 NS1 cells on their contralateral flank. Another 10 animals were de novo inoculated control animals, not previously included in the study. All de novo inoculated control animals developed tumors. In the previously cured animals, no tumor growth could be detected during the observation period of additionally 100 days. Survival was significantly increased in all the cured animals as compared to the de novo inoculated controls. At 14 days after inoculation, there was no statistically significant difference in tumor size in the de novo inoculated controls in the re-challenge series compared to the controls in the first series, indicating a stable tumor growth in control animals.

Increased survival but no cure in animals with intracranial glioblastoma irradiated with FLASH 12.5 Gy × 2 or CONV-RT 12.5 Gy × 2

Survival was significantly increased in the irradiated animals versus control animals. There was no difference between the two irradiation modalities.

Serum analytes from animals treated with CONV-RT, FLASH and controls

Serum was collected from cured animals and compared to that of control animals which had been euthanized due to large tumors. Serum levels of GM-CSF, ICAM-1, IL-2, IL-6, IL-18, TIMP-1, TNF-alpha and VEGF were compared between animals.

If serum expression was below or above technically detectable levels, it was reported as out of range. Comparing long-term survivors that had been treated with FLASH 8 Gy × 2 or CONV-RT 8 Gy × 2 demonstrated that there was no difference between these groups of animals regarding any of the serum analytes. However, TIMP-1 levels, were significantly reduced in animals that had been treated with FLASH 8 Gy × 2 compared to control animals. In animals with subcutaneous tumors, TIMP-1 was reduced in FLASH treated animals compared to control animals.

Gene expression

Since we in this study were able to cure some animals in the subcutaneous model with radiotherapy and achieve long term anti-tumor immunity, yet intracranial tumors could not be cured, we wanted to explore different tumor properties in the intracranial versus subcutaneous setting.

Tumor samples with insufficient RNA quality had to be removed. In the final analysis one intracranial control tumor was compared to five subcutaneous control tumors.

There was a total number of 219 differentially expressed genes between the intracranial and subcutaneous control tumors, out of a total of 32,883 included genes in the array. Interestingly, most of the genes with the highest fold change were associated with immune response. CD74 was among the differentially expressed genes with highest counts (> 1000 counts per million in all animals, fold change 3.44, p = 2.11E−09)

All of the most differently expressed genes were upregulated in subcutaneous controls versus intracranial control animals.

Paper II

Comparable Long-Term Tumor Control for Hypofractionated FLASH Versus Conventional Radiation Therapy in an Immunocompetent Rat Glioma Model

Survival was significantly increased in all irradiated groups compared with control animals. No statistically significant difference between animals treated with FLASH-RT and CONV-RT at any of the dose levels. For CONV-RT, there was a statistically significant difference comparing 8 Gy × 3 versus 12.5 Gy × 3 or 8 Gy × 3 versus 15 Gy × 3, but no difference comparing 12.5 Gy × 3 versus 15 Gy x 3. For FLASH-RT, there was a statistically significant difference comparing 8 Gy × 3 versus 15 Gy × 3 but no difference comparing 8 Gy × 3 versus 12.5 Gy × 3 or 12.5 Gy × 3 versus 15 Gy × 3.

Treatment response

45 of 58 of the irradiated animals were alive on day 100, and in 23 of 58 a long-term tumor control was achieved by RT. For animals irradiated with 8 Gy × 3, a similar tumor control was observed for CONV-RT and FLASH-RT, with 4 of 9 and 4 of 10 animals with long-term tumor control or stable disease on day 100, respectively. For animals irradiated with 12.5 Gy × 3, 2 animals in the FLASH-RT group were euthanized before the end of the study due to large tumors, whereas long-term tumor control or stable disease was evident for all animals in the CONV-RT group. All animals irradiated with 15 Gy × 3 achieved long-term tumor control or had stable disease, except for 1 animal belonging to the CONV-RT group, which was euthanized 1 week before the end of the observation period due to a large tumor.

Radiation-induced skin reactions

Skin reactions were overall mild and consisted of mainly hair loss, erythema, and dry desquamation. No severe toxic effect (grade >4) was observed. No significant difference in acute side effects between FLASH-RT and CONV-RT for any of the investigated dose levels at any of the investigated time points. A majority of the acute side effects healed spontaneously. Late side effects greater than grade 1

increased with increasing fraction dose. No significant difference in late side effects was seen between CONV-RT and FLASH-RT for any of the dose levels investigated.

Paper III

Comparable Survival in Animals with Intracranial Glioblastoma Irradiated with single-fraction Conventional Radiotherapy or FLASH Radiotherapy

In this study we wanted to explore whether we could achieve an effective irradiation dose in animals with intracranial glioblastoma irradiated with CONV-RT versus FLASH-RT in one fraction. As stated above, in paper I and II we managed to prolong survival for intracranial glioblastoma, but did not achieve long-term cure at the dose 12.5 Gy x 2 [269]. Here we explored single doses of 20 Gy, 25 Gy and 30 Gy. Since an optimal fractionation has not yet been studied sufficiently in the FLASH-RT model, we decided to first establish an optimal dose with one fraction.

49 animals were included in the survival study, four additional animals were euthanized on day 7 without receiving any treatment and the brains were evaluated for tumor size. They all had detectable small tumors. Survival was significantly increased in animals irradiated with FLASH-RT at 20 Gy as well as CONV-RT at 20 Gy compared to control animals. Survival was also significantly increased in animals irradiated with FLASH-RT 25 Gy as well as CONV-RT 25 Gy compared to control animals. Survival was not increased in animals irradiated with FLASH-RT 30 Gy or CONV-RT 30 Gy compared to control animals.

The longest overall survival was reached in the animals irradiated with FLASH-RT at 25 Gy, with a mean overall survival of 60 days, followed by those irradiated with CONV-RT 25 Gy with a mean overall survival of 55 days.

Control animals all had tumors of intermediate size or large size. Tumor size differed significantly between the groups upon euthanasia. No animal treated with 30 Gy of either modality displayed large tumors occupying more than half of the hemisphere. One animal irradiated with FLASH-RT at 25 Gy had no measurable tumor at the end of the study and survived 100 days. One additional animal that had been irradiated with FLASH-RT at 25 Gy died on day 49, without any visible signs of large tumor. The other animals irradiated with FLASH-RT at 25 Gy or CONV-RT at 25 Gy had intermediate or large tumors.

A significant negative correlation between tumor size and irradiation dose were seen when all animals were included.

Dermal side effects

Based one previous observation done in paper I and II the dermal side effects were only evaluated two weeks after irradiation and, in the animals, irradiated with 30 Gy or 20 Gy. Once again dermal side effects were mild, and only consisted of hair loss in some of the animals. FLASH-RT irradiation at 20 Gy led to no dermal side effects, in contrast to CONV-RT at the same irradiation dose where hair loss was observed. Evaluation at day 100 after tumor inoculations was not performed, due to the small remaining sample size in each group

Paper IV

Combined anti-C1-INH and radiotherapy against glioblastoma

In this paper the aim was to explore the combination of anti-C1-INH antibodies with radiotherapy in two different tumor microenvironments. Once again, the chosen radiation dose of 8 Gy × 2 was based on the results presented above in papers I-III. Reduced numbers of fractions and increased dose per fraction seemed to be beneficial in regard to immune system activation [138]. The dose of anti-C1-INH was based on a previous study performed by our group [128], where we could demonstrate increased survival as a result of intratumoral treatment in subcutaneous tumors. The dose, however, was reduced in the present set-up, since we wanted to determine the possible interaction with radiotherapy. Thus, the individual therapies were delivered at sub-therapeutic doses and levels.

Increased survival in irradiated animals but no added effect of anti-C1-INH in animals with intracranial glioblastoma

23 animals were included. Animals were treated with irradiation and immunotherapy, and divided into four groups as follows:

1. Control animals with tumor inoculations but no further treatment (n = 6)
2. Animals treated with RT 8 Gy × 2 (n = 6)
3. Animals treated with intratumoral anti-C1-INH (n = 6)
4. Animals treated with both RT 8 Gy × 2 + anti-C1-INH (n = 5)

Survival was significantly increased in animals treated with radiotherapy (RT 8 Gy × 2 versus control $p = 0.007$). Anti-C1-INH together with radiotherapy also increased survival significantly, but there was no synergistic effect of adding anti-C1-INH to radiotherapy. There was no significant effect of anti-C1-INH as stand-alone therapy. Increased concentration of intratumoral anti-C1-INH did not yield any increased effect compared to control animals delivered as single therapy.

Gene expression analysis

The expression of C1-INH was reduced in animals treated with anti-C1-INH compared to control animals. C1-INH expression was increased in irradiated animals, although no statistical significance was reached. Raising the question if irradiation could further sensitize the animals for anti-C1-INH treatment by increasing the C1-INH expression.

C1r, C1s, C2, C3, C4b, CD55 differed between groups. C1r, C1s, C2, C3, C4b were increased in animals treated with combination of RT and anti-C1-INH when compared to control tumor. Although down-stream components of the classical pathway were not increased. CD55 was increased in the same treatment group when compared to tumors from control animals. CD55 (decay-accelerating factor, DAF) is known as one of the regulators of the complement cascade [277]. CD55 is expressed on nearly all cells of the body and are commonly overexpressed on tumor cells [277].

In animals treated with RT and RT together with anti-C1-INH, Serpine2 was reduced compared to control. A high expression of Serpine2 has been seen to escalate cellular migration and proliferation, at least in squamous carcinoma, and has been suggested as a cancer-promoting factor that increases angiogenesis [278]. Its particular role in glioblastoma is not yet known it seems however, to be plentiful in glioblastoma, compared to meningioma where it is very sparse [279].

Increases of Igf2 was seen after treatment with anti-C1-INH, and in combination with RT and anti-C1-INH when compared to control.

Igf2 has been suggested as a possible neuroprotective agent and it has been speculated to be a treatment for neurological disorders such as Alzheimer's disease and Huntington's.[280]

Increased in all treated groups was Thbs1, which has been shown to be upregulated in high grade gliomas and has been associated with poor prognosis [281]. Possibly, it is an important factor for tumor progression, despite treatment and therefore an interesting target for future research into possible therapies.

Increased survival after combined treatment with anti-C1-INH and irradiation in animals with subcutaneous glioblastoma

From the intracranial experiments RT combined with anti-C1-INH altered many of the upstream genes of the classical pathway of the complement system, but, with no significant alterations of the downstream components. We saw no signs of synergistic or additional effect of adding anti-C1-INH to RT when it came to survival.

In our subcutaneous model the dose of anti-C1-INH could be increased and with no blood–brain barrier we were hoping to see an increased efficacy of our immune therapy.

28 animals were included and divided into groups.

1. Control animals (n = 5)
2. Animals treated with RT at 8 Gy × 2 (n = 6)
3. Animals treated with intratumoral anti-C1-INH (n = 6)
4. Animals treated with RT at 8 Gy × 2 + anti-C1-INH (n = 5)
5. Animals treated with PBS intratumorally (n = 6).

We added intratumoral PBS to rule out that just injecting an extra volume of PBS would disrupt the tumor growth. Animals that were euthanized prior to the end of the study at day 100 were done so due to tumor growth exceeding the maximally allowed diameter of 30 mm.

Survival differed significantly between groups. Compared to control animals, survival was significantly increased in animals treated with RT + anti-C1-INH versus control animals whereas the other groups did not differ significantly from the control animals. Animals treated with combined RT + anti-C1-INH had 60% long-term survivors, still alive at day 100 after tumor inoculations.

Tumor size differed significantly between the groups. Compared to control animals, tumor size was significantly reduced in animals treated with RT + anti-C1-INH, whereas the other groups did not differ significantly from the control animals.

The immunohistochemical expression of CD8, CD4 and FOXP3 was compared in animals that were euthanized on the same day. Both irradiated and control animals generally exhibited relatively intense CD8 positivity, except necrotic areas, where there was no specific staining. Staining with CD4 and FOXP3 was detectable, but sparse in comparison. From an immunohistochemical point of view, no pattern could be defined that clearly distinguished the control tissue from the irradiated tissue.

Discussion

Is FLASH-RT a potential treatment option in glioblastoma?

We set out with the intention to provide additional support in the new exciting field of FLASH-RT and its promises. We have been able to demonstrate that FLASH-RT and CONV-RT have equal anti-tumoral effects in an experimental glioblastoma model. When it comes to long-term immunity, they also seem to be comparably effective in generating such a response.

In common for all our findings in paper I-III was that, despite previous results from *in vitro* studies of sparing of tumor cells in FLASH-RT [214], we saw no difference when comparing the two modalities regarding survival. This was true for all doses, spanning from 8 to 25 Gy (at 30 Gy we saw no significantly increased survival compared to control for either modality), as well as both intracranial and subcutaneous models. In accordance with previous studies by Montay-Gruel et al and Bourhis et al on glioblastoma animal models [196, 202] we could also see a delayed tumor growth in the irradiated animals. In paper II we compared hypofractionated FLASH-RT versus CONV-RT as treatment for large subcutaneous glioblastomas. All animals had verified tumors upon initiation of treatments. Doses in this paper were chosen to achieve tumor growth delay as well as high probability of long-term tumor control. In this paper however, animals irradiated with doses of 8 Gy × 3 proved to be insufficient for tumor control in all animals for both FLASH-RT and CONV-RT. But higher doses attained long-term tumor control or stable disease on day 100 in the majority of the animals. In Montay-Gruel et al's study no animal with intracranial glioblastoma irradiated at 10 Gy × 3 lived for 100 days, important differences are that this mouse model used in their study was immunodeficient and intracranial, where in paper II we utilized our subcutaneous model in fully immunocompetent animals. [196]

In paper I we could demonstrate a long-lasting anti-tumor effect in our subcutaneous model, using both CONV-RT and FLASH by showing that the cured animals managed to reject tumor cells inoculated on their contralateral side. *In vitro*, a dose–response could be demonstrated with both modalities, showing a reduction of colony formation in relation to increased doses. A difference was seen at 18 Gy,

where CONV-RT was more efficient compared to FLASH. Further increases resulted in no detectable colonies.

Animals in the subcutaneous model treated with FLASH at 8 Gy × 2 showed significantly lower levels of TIMP-1 compared to control animals. Low tumor immunohistochemical expression of TIMP-1 has been seen to be associated with significantly longer survival in human patients when compared to high TIMP-1 expression. TIMP-1 may have several roles that promote tumor growth and invasion. [282] Even while receiving higher irradiation doses none of the animals in the intracranial trial reached beyond day 40. While comparing the genes one of the most upregulated ones was CD74 in the intracranial setting, a cell membrane receptor of cytokine macrophage migration inhibitory factor (MIF). It is known to protect against injury and promote healing in several organs. [283] Inhibition of CD74 has been proved to diminish tumor growth in different cancers [284, 285] as well as restoring anti-tumor activity of immune cells in melanoma [286]. CD74 is known to be expressed in gliomas and more so in high-grade gliomas compared to low-grade. Xu et al showed in their study that a high expression of CD74 was associated with poor prognosis and high immune infiltration. [287] Further studies indicate that CD74 and MIF interaction is associated with the microenvironment of gliomas and could be facilitators in the proliferation process. [288, 289] Kitange et al saw signs of CD74 being one of the contributing factors to Temozolomide resistance in their study from 2010 both in vitro and in vivo on nude mice. [290]

Intracranial setting

As explained previously, the dose was increased in the intracranial setting based on previous findings in our group [137]. Although both modalities once again prolonged survival compared to control animals, cure could not be achieved in the intracranial setting, even with the dose-escalation compared to the subcutaneous trial. Irradiation was started on day 9 after tumor cell inoculations. Based on previous imaging done on the cell-line NS1 with MRI, where no detectable tumor was seen at 7 days after intracranial inoculations, and only a very small tumor on day 10, this should not be an issue related to too large tumors at that time [268]. Our findings of long-term anti-tumor effects and cure after irradiation further supports the importance of radiotherapy in the battle against cancer in not only the local setting but also for an immunological response, for example the abscopal effect. This is one of the many reasons behind the interest in combing radiotherapy with immunotherapy. [135-138, 291].

Glioblastomas development of adaptive radioresistance is a challenge. As discussed previously in the introduction of this thesis, there are several contributors to radioresistance in GBM. [165] The findings in paper I further supports the theory regarding the microenvironment's involvement in this development since the tumors in the intracranial setting proved to be much more radioresistant compared to those

in the subcutaneous setting. [292] Apart from this the blood-brain barrier also needs to be taken into consideration when trying to understand and explain the difference in survival. Although the BBB is partly disrupted in the GBM affected brain it is still not a free passage. [99] What is also known is the fact that radiotherapy additionally can disrupt the BBB by increasing permeability. [293, 294] Further evidence that this phenomena was immune driven was put forward by Blethen et al in 2023 where they studied the effects on both an immunocompetent model as well as an immunocompromised model. [295] To gain a more comprehensive understanding of this mechanism it would be helpful to improve the timing of additional anti-tumor therapies that might benefit from a disrupted BBB to enter the CNS, but also to elicit a stronger immune response. FLASH-RT is yet to be explored in the Stupp protocol of 60 Gy/30 fractions used for glioblastoma patients. To enable the future clinical translation of FLASH-RT the therapeutic window should be studied in models where tumor cure can be achieved. Although our subcutaneous model cannot be used to draw conclusions about the intracranial microenvironment, we have utilized it to investigate tumor control and normal tissue complications.

Since our model is based on fully immunocompetent animals, with an aggressive intracranial tumor, all control animals that received tumor cell inoculations but no further treatment, died due to tumor growth. In paper III we saw that a single fraction of CONV-RT versus FLASH-RT at 20 and 25 Gy resulted in comparable survival. In this paper we analysed the brains of the animals with immunohistochemistry with focus on quantifying the tumor size and to determine if the animals indeed died due to large intracranial tumor burden. Since NS1 express GFP it is easy to evaluate even smaller tumors or satellites with anti-GFP immunostaining. In paper III we saw that animals irradiated with 30 Gy with FLASH-RT or CONV-RT, did not die with large tumors, but did not have an increased survival. In contrast those irradiated with 20 Gy, the majority of the animals died with massive tumors, indicating that the dose of 20 Gy x 1 was too low to achieve sufficient tumor control in those cases. This suggests that the irradiation dose had been too high at 30 Gy in relation to the acute radiotoxic effects that the animals could tolerate.

Side effects

Skin toxicity

In paper II we explored dermal side effects in relation to radiotherapy. We could see that the local early side effects were time dependent. However, despite previous tissue saving finds regarding FLASH-RT, we could show no difference between FLASH-RT and CONV-RT regarding both acute and late side effects in any of the investigated dose levels. Previous studies exploring skin toxic effects [193, 221, 296-298] suggest that it might be necessary for higher doses in order to achieve a FLASH-sparing effect in the skin. For example, Vozenin et al administered single

fractions in the range of 28 to 34 Gy the skin of a pig in their study that yielded the result of minimized skin toxicity in FLASH-RT. [193] Soto et al explored doses of 30 and 40 Gy when examining skin toxicity and found a sparing effect of FLASH-RT in this span in comparison to CONV-RT, but no severe toxicity was found at doses below 20 Gy. [296] Gaide et al studied a human patient with relapsed cutaneous T-cell lymphoma irradiated with 15 Gy FLASH-RT and 15 Gy CONV-RT and did not observe any difference in acute or late effects. [296, 297]

In paper II a possible limitation is the impact that a larger subcutaneous tumor could have on our scoring of dermal side-effects. It is therefore important to remind the reader of the ethical guidelines where all animals with tumors larger than 30 mm were euthanized and at the time of long-term evaluation. No animal had larger tumors that would affect the scoring. Although it would still be beneficial for future studies to evaluate toxicity in a non-tumor bearing model.

Skin toxicity is also an important factor to consider in relation to wound healing complications in the clinical setting, with ulcers resulting in infections that may lead to long-term issues for the patients [299]. For glioblastoma a too early initiation of radiotherapy might increase the risk for surgical site infections, but as we know concerning glioblastoma time is also of the essence due to its rapid growth [300]. In paper III we did an early evaluation of dermal side effects at two weeks post-irradiation. To put this into perspective the animals underwent radiotherapy only seven days after surgery, no severe dermal side effects were seen at the area of the skin incision.

Neurocognition

As presented in the background there are several hypotheses surrounding the mechanisms behind radiation-induced damage in the CNS [149, 156]. Neuro-inflammation leading to elevated oxidative stress, change in neurons transmitting abilities, loss of dendritic spines and damage to the blood-brain barrier leading to ischemia and neuro-toxicity [149]. Soffietti et al points out in their review from 2023 there has been a lack of animal models that adequately mimic the clinical situation. Most notably some of these models lack brain tumor all together or have received whole-body radiation instead of targeted brain irradiation, neither which represents the current protocols or the actual clinical reality. [149]

Montay-Gruel et al studied FLASH-RT in their nude mice model and could demonstrate improved neurocognitive function following brain irradiation with hypofractionated 10 Gy × 3 FLASH-RT compared with CONV-RT at equal doses. [196] They also showed that FLASH-RT led to reduced reactive gliosis in the brain compared to CONV-RT, which potentially might contribute to the FLASH effect seen. [198]

In future studies, it would be interesting also to explore cognitive side effects in our model, as well as effects of fractionation and dose. As mentioned above Montay-

Gruel et al saw sparing effects of FLASH-RT in doses of 10 Gy x 3, as compared to CONV-RT at the same dosage [196]. For us to make this happen and have a reasonable statistical power we need more long-term survivors of animals with intracranial tumors, therefore we need to find an irradiation dose resulting in an even higher degree of survival.

The FLASH effect

In accordance with previous statements regarding the FLASH effect it is probable that the dose for inducing a FLASH-sparing effect varies between tissue types. Although the exact mechanism is still unknown, it is likely affected by several factors such as oxygen concentration, iron levels, DNA-repair ability and effects on the immune system [157, 190, 204, 211]. Since our animal model is fully immunocompetent this might also be a factor to consider in the response to radiotherapy. As discussed extensively in the introduction, radiotherapy does not only perform a local anti-tumor effect, but it is now well established that it has several effects on the microenvironment as well as the immune system in large. [301] One of the theories behind the FLASH effect is its effect on the immune system, mainly being that due to shorter time with FLASH, fewer lymphocytes are irradiated, thus leading to less lymphopenia, a known side-effect of radiotherapy. [180, 218, 219] This strengthens our belief that it is of outmost importance to investigate the FLASH effect in immunocompetent animal models to further decipher its underlaying mechanisms.

Treatment parameters previously recognized as critical for the FLASH effect are average dose rate, instantaneous dose rate, beam-on time, and fraction dose. [191, 202, 205] It should be noted that similar temporal parameters have been used leading to a sparing FLASH effect *in vitro*. [204, 214] The absorbed dose was measured before each treatment session and confirmed that the prescribed doses were delivered accurately. The temporal structure of the electron beam has been shown to be important in FLASH-RT. [191, 202, 205]

Is the complement system an interesting target for radio-immunotherapy?

In paper IV we saw a long-term anti-tumor effect in animals treated with the combination of radiotherapy and immunotherapy with anti-C1-INH in our subcutaneous GBM model. Interestingly, the treatments as stand-alone therapy did not prove to be successful as delivered in that study. Size of irradiation field was decreased in comparison to animals with subcutaneous tumors in paper I [269]. The doses of anti-C1-INH were reduced in comparison to previous protocols done by

our lab by Fornvik et al from 2018 [128]. Once again it is of great strength that this study was performed in our fully immunocompetent animal model.

As stated above this anti-tumor effect was seen in our subcutaneous model, unfortunately the same treatment was not found to be equally effective in the intracranial model. Perhaps because we must decrease the doses of anti-C1-INH, due to the limited volume we are able to inject intracranially, the dose was simply too low to in this case to generate the same suggested strong immunological response. This of course leads to the question of administering anti-C1-INH through for example intravenous administration. Our thoughts regarding this approach are that there might then be a need to initially neutralize circulating anti-C1-INH, before effects on the tumor could be achieved. This in turn might increase the risk of side effects. Another option would be to try a local delivery of anti-C1-INH, for example with intratumoral osmotic pumps.

We could see a down-regulation of C1-INH because of the anti-C1-INH treatment intracranially in our gene analysis, but not of statistical significance. We could only however include samples with sufficient RNA quality in the gene expression analysis, and unfortunately many samples did not pass the qualification needed, which might have altered the results. In the treatment group we could see that up-stream mediators of the classical pathway of the complement system were increased.

As presented earlier in this thesis the complement system might have different roles regarding cancer, but also in the aspect of when utilized as anti-tumor treatment, concentrations might decide its role. [277] Gunn et al showed in their study from 2012 that tumor cells with C5a, could lead to both increased and decreased tumor growth, depending on C5a expression. In the complement cascade C5a is an important immune cell mediator, it interacts with the C5a receptor, which promotes vasodilatation and thus increased infiltration of immune cells to the tissue, such as NK cells and macrophages, which showed a reduction of tumor growth [277].

Once again a contributing factor to the differences seen in the intracranial setting is of course the different microenvironment but also the blood–brain barrier (BBB), which contributes to the CNS's immune privileged site [99]. As stated in the background although the BBB is partially disrupted in glioblastoma, tumors cells can still hide behind an intact barrier. We have discussed different approaches to further circumvent the BBB. One approach is the use of focused ultrasound (FUS). It has been demonstrated that FUS can open the BBB and lead to an increased delivery of systemic therapy to CNS, with no major side effects detected [302]. Wang et al could successfully deliver neurotrophic across the BBB with MRI guided FUS [303]. Since we saw an increased efficacy of combining our immunotherapy and radiotherapy in the subcutaneous setting, but not in the intracranial setting, it would be of outmost interest to explore whether by opening the BBB with FUS or a similar method would yield a more beneficial response, especially in combination with applying concomitant radio-immunotherapy.

A reoccurring discussion is the timing of RT to induce the most optimal immunological response and understanding the role it plays. Studies performed in other cancer models show that the timing of RT and immune checkpoint blockade indeed seems to matter. Dovedi et al showed that PDL1 was upregulated 24–96 h post RT and when delivering PDL1 blockade 7 days after RT no survival benefit was seen, compared to administration on day 1 or 5 that improved survival [304]. This further supports that timing is of the essence in the response of radio-immunotherapy and might affect the outcome in our model as well using anti-C1-INH.

Apart from the apparent differences in tumor microenvironment, these studies have raised the question about the effects of an intraoperative approach, combatting the BBB. One such method is intraoperative radiotherapy. For this method, in the same manner as FLASH-RT, a high single-dose of radiotherapy is administered to the tumor bed, while healthy tissue is shieled of, as described by Calva, Meirino and Orecchia in their 2005 review of the subject. [305] This method is also reviewed in the ongoing INRTAGO-trail [306], the rationale behind the trial refers to previous research showing a more beneficial systemic and local response to high single doses in comparison to conventionally fractionated RT. [307, 308]

This reasoning is well in line with the ongoing findings regarding FLASH-RT. The trial is a prospective trial with primary objective to define the tolerated dose of this radiotherapy and furthermore as secondary objective to evaluate progression-free and overall survival for the patients. In 2019 Giordano et al published early data from the trail with 15 patients, showing a manageable toxicity. The INTRAGO I trial was a phase I/II trial and INTRAGO II is now ongoing as well as a phase III trial for progression free survival of patients with newly diagnosed GBM. [309] Previous reviews, pooled- and meta-analyses of the method suggest a tolerable complication ratio and comparable survival rates with current treatment regimens and sometimes even superior. The ongoing INTRAGO II will be instrumental in whether this method should be added as a possible treatment option in the future. [310-312] What intrigues me in this aspect is the added possibility of making this more manageable with FLASH-RT, although still short time with the current radiotherapy, FLASH would reduce the timeframe even further. Yet, since this is not yet in clinical practice the timeframe of actual clinical implementation would be delayed.

On the same spectra is the review by van Solinge et al from 2022, “Advances in local therapy for glioblastoma — taking the fight to the tumor” [313], where they bring up a number of interesting emerging approaches. Regarding radiotherapy, they bring up studies focusing on brachytherapy, where iodine seeds were implanted. Although proving safe, no additional benefit was proven. What was also of notice is the tolerability for patients to receive brachytherapy, although most patients already had undergone treatment with radiotherapy. This is also one of the cornerstones in the prospects of FLASH-RT, if it is less damaging to the surrounding

tissue it would open up for more possibilities of re-irradiation for the patients. Radiotherapy against GBM is effective but limited in reoccurring GBM, and as van Solinge et al states in their review, GBM mostly reoccurs in or near the original site. Meaning that the irradiation doses on the surrounding healthy tissue is now considered too harmful. They also bring up local immunotherapy, mostly viral therapy, cytokine therapy and immunostimulant therapy. [313]

On this theme is also the interest of other locally administered drugs, discussed in detail by Gazaille et al in their review from 2021, where nanoparticle-loaded hydrogels stood out as especially interesting, partly due to their multi-target possibility, which as we know is essential in the fight against GBM and its heterogeneity on all levels. [314] A drawback with this approach is the need of surgery, although many, but not all GBM patients undergo surgery. This was also published prior to recent findings of effects of CAR-T therapy on solid tumors and GBM, such as Bagley et al's bi-valent CAR-T therapy which is ongoing. [264]

Conclusions

In paper I we could demonstrate equal efficacy of CONV-RT and FLASH in our fully immunocompetent glioblastoma animal model. Radiotherapy was extremely efficient in the subcutaneous setting, leading to cure in most of the animals. In cured animals, a long-term anti-tumor effect could be seen. Unfortunately, the same was not seen in the intracranial setting, perhaps due to radioresistance or too low doses.

In paper II we continued exploring long-term tumor control and showed that this could be achieved in large subcutaneous glioblastomas without severe skin toxicity, using both hypofractionated FLASH-RT and CONV-RT. We saw no difference in tumor response between the two modalities. No major treatment toxicity was found, suggesting that we might need to administer higher doses to see the FLASH effect. This study is however the first to show that there is no difference in long-term tumor control rates between FLASH-RT and CONV-RT in immunocompetent glioblastoma animal model.

In paper III we explored whether a single-fraction of CONV-RT and FLASH-RT were equally effective against intracranial glioblastoma in the same fully immunocompetent model. Animals irradiated at 30 Gy x 1, the highest examined dose, did not die due to large tumor burden, whereas those irradiated with lower doses had larger tumors at the time of euthanasia.

In paper IV we demonstrated that anti-C1-INH treatment combined with radiotherapy could increase survival in our subcutaneous glioblastoma model. In the intracranial setting no effect was seen of anti-C1-INH treatment. This could have several explanations but perhaps the most important factor may be too low doses

and the additional role of the BBB, as well as the difference in immunological response in the brain compared to the subcutaneous setting.

Moving forward

As briefly mentioned, it is of great interest to further explore the neurocognitive effects of FLASH-RT in our model and this work is already ongoing. We have examined non-tumor bearing Fisher rats that have received FLASH and CONV RT, with both NOR-testing and immunostaining, and are currently extending these trials.

Regarding the FLASH effect in general I believe we can conclude that higher doses might be needed, although as apparent at least in our findings in paper III a single dose of 30 Gy for example, proved to be too much in the intracranial setting, using the settings described in our study. This further implicates the importance of finding the appropriate doses needed in different animal models before we are able to move towards clinical translation of FLASH-RT. Our studies have not demonstrated any additional side-effects compared to CONV-RT, at least showing promise for it to be a safe method in that aspect. Even more importantly, we have seen that the FLASH-RT does not seem to be tumor sparing in our model compared to CONV-RT, which otherwise could have been a major problem. FLASH-RT is delivered much faster than CONV-RT. A concern regarding FLASH-RT is to be able to reach deep sited structures, which depends on the modality of FLASH-RT that is used. A major strength in the works lay in our choice of an immunocompetent animal model with a highly aggressive and infiltrative tumor.

The further complications related to the complicated nature of the brain and glioblastoma are also important factors that are in need of both more basic science to understand the mechanisms on a deeper level, but also pre-clinical studies where we continue to try to combat its illusive nature. It brings me much joy to read about new novel approaches such as Marrocco et al's imaginative nose-to-brain drug delivery [210] as well as Bagely et al's bivalent CAR-T cells treatment [264]. Because when battling the ever changing and illusive glioblastoma multiforme it is my personal belief that it is this type of ingenuity and continued strive to push forward that will one day lead to the cure.

To conclude, it is this authors hope, that although far from comprehensive, this thesis still has aided in pushing the research of this diagnosis a bit further and that it will inspire future work in the field.

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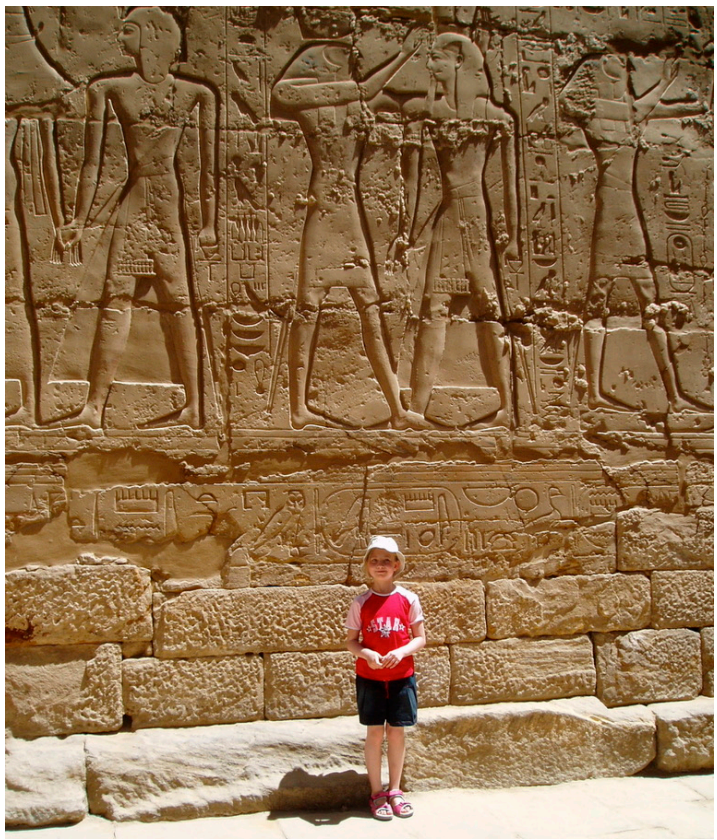

And to 10-year-old me: I hope you are proud, even though it's not a PhD in Archaeology.

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EMMA LILJEDAHL, MD, history buff, true-crime enthusiast, childless cat lady, and self-proclaimed Swiftie. My research focuses on glioblastoma, our deadliest primary brain tumor.

Glioblastoma remains a major challenge despite advances in cancer research. Radiotherapy is essential, but its side effects on healthy tissue are a significant limitation. In my thesis, I explore whether FLASH radiotherapy can reduce these side effects while maintaining anti-tumor efficacy, comparing it to conventional radiotherapy. I also investigate how activating the complement system might enhance treatment outcomes.

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