Tumor heterogeneity in glioblastoma

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a real-life brain teaser

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PROEFSCHRIFT

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Chapter 1

Introduction

Introduction in GBM

Glioblastoma (GBM) is the most common malignant primary brain tumor occurring in approximately 3 per 100.000 adults per year [1]. Most GBMs develop without a known etiology, with only a minor fraction linked to specific risk factors (i.e., ionising radiation exposure to the head and neck) or familial predispositions [2].

The current standard-of-care for GBM patients consists of resecting the tumor when the location of the tumor allows it, followed by concurrent chemoradiation and adjuvant chemotherapy using temozolomide (TMZ) [3]. Unfortunately, despite this multimodal treatment, a 5-year overall relative survival of only 6.8% is achieved [4]. This dismal prognosis, combined with the establishment of the standard-of-care regimen more than fifteen years ago, shows the urgent need to improve treatment options for GBM patients.

Evolutions in glioblastoma diagnostics

Despite the lack of substantial changes in the current treatment schedule for GBM, significant developments have been made in optimising GBM diagnostics using radiological and histopathological methods.

Magnetic resonance imaging (MRI) is the most commonly used diagnostic modality for GBM. GBM is characterised as a contrast-enhanced tumor with a necrotic core surrounded by non-enhancing signal abnormalities, consisting of edema and microinfiltration of tumor cells[5]. Recent advances in MRI, such as diffusion-weighted imaging (DWI), perfusion-weighted MRI and positron-emission tomography MRI (PET-MRI), are increasingly used in clinical practice [6]. DWI has a high sensitivity to detect early ischemic injury and infection/abscess and is therefore used to discriminate between these pathologies and GBM [7]. Perfusion-weighted MRI can measure microvessel density and distinguish GBM from other tumor types[8] or lower-grade gliomas [9]. Amino-acid PET tracers can differentiate GBM from other brain pathologies and between actual tumor progression and radiation necrosis [10].

Whilst MRI suggests the initial GBM diagnosis, histopathological analysis of tumor tissue remains the golden standard for definitive GBM diagnosis. Previously, GBM was diagnosed based on histological features alone. The pathological hallmarks of GBM are diffuse infiltration of tumor cells with astroglial appearance, microvascular proliferation and/or pseudopalisading necrosis [5].

This changed due to the implementation of the 2016 [11] and subsequent 2021 [12] update on the World Health Organization (WHO) classification of central nervous system (CNS) tumors in which histological and molecular features were integrated [11]. In daily clinical practice, several molecular markers are now implemented in the diagnostic work-up of GBM. Isocitrate dehydrogenase (IDH) mutations (most commonly the IDH1 R132H mutation) are associated with younger age, improved prognosis and (dedifferentiated) low-grade gliomas [13]. In the new WHO classification, IDH wild-type tumors that were previously histologically classified as low-grade glioma are still considered as GBM in the presence of either telomerase reverse transcriptase (TERT) promotor mutations, the combined gain of chromosome 7 and loss of chromosome 10 or epidermal growth factor receptor (EGFR) amplification.

Another molecular marker integrated into the clinical diagnostic workup is the combined deletion of chromosomes 1p and 19q. This is used to discriminate oligodendrocyte tumors from astrocytic tumors and is never found in GBM [11].

The only established predictive molecular biomarker is promotor methylation of the methylguanine methyltransferase (MGMT) gene. MGMT is involved in the repair of DNA damage induced by alkylating chemotherapeutics, such as TMZ and lomustine. Lack of MGMT protein expression due to gene methylation impairs DNA repair in tumor cells and is therefore associated with a more favorable response to TMZ [14].

Additionally, new approaches such as DNA methylation-based classifiers have been developed for a more accurate diagnosis [15]. The utility of such classifiers is, however, currently still limited to clinical research or difficult to diagnose cases.

The (failed) attempts in improving glioblastoma treatment

Even though the previously described STUPP regimen has remained the standard-of-care treatment for GBM patients for over fifteen years, numerous attempts on testing novel treatment options have been conducted [5]. The only newly approved treatment for GBM is tumor treatment fields (TTF) [16], which remains infrequently used in GBM [17]. TTF uses electric fields to induce antimitotic effects as well as to interfere with other biological processes such as DNA repair, autophagy and cancer cell migration [18]. In addition, multiple systemic anti-cancer treatments showed initial promising results, both in preclinical and clinical studies. However, none of them succeeded in showing a benefit in overall survival (OS) in phase III clinical trials [19].

Bevacizumab, a monoclonal antibody directed to the vascular endothelial growth factor (VEGF) receptor, is approved by the FDA to treat recurrent GBM. The actual

benefit of bevacizumab is, however, controversial, as the observed improvement in progression-free survival (PFS) can potentially be attributed to radiological 'pseudo-response' due to its antiangiogenic properties causing blood-brain barrier (BBB) stabilisation and decrease in tumor volume on MRI without an actual anti-tumor response. The lack of benefit on overall survival was confirmed by multiple studies [20-22]. Therefore bevacizumab has not been approved for GBM patients in the European Union and is only used to treat severe radiation necrosis.

Improving GBM treatment has mainly focused on targeting molecular alterations commonly found in GBM. EGFR amplification is the most common genetic alteration in GBM, occurring in about 50% of all cases, and therefore, EGFR is one of the most extensively studied targets [23]. A subset of EGFR-amplified GBM harbours the specific EGFRvIII mutation, a deletion of exon 2 to 7 specific to GBM. Suppressing EGFR activity by using tyrosine kinase inhibitors [24], targeting neoantigen EGFRvIII using a peptide vaccine [25], or using an antibody-drug conjugate that binds specifically to EGFR amplified cells [26] all have failed to exhibit clinical success [5]. These failures could be contributed to inadequate patient selection in clinical trials, to the subclonal presence of EGFR mutations and its elimination at recurrence as well as to compensatory upregulation of other intracellular pathways.

Apart from EGFR, multiple molecular pathways implicated in tumorigenesis of GBM have been targeted with small molecule drugs and antibodies. These include, but are not limited to, fibroblast growth factor receptor (FGFR), MET, platelet-derived growth factor receptor (PDGFR), cyclin-dependent kinase (CDK), mitogen-activated protein kinase kinase (MEK), BRAF, and mammalian target of rapamycin (mTOR) [5]. However, to date, none of these targets have made it into FDA-approved drugs for GBM.

Besides directly targeting GBM cells, utilising the tumor micro-environment (TME) to induce an anti-tumor response has also been studied. Exploiting the immune system by using immunotherapy approaches has caused a revolution in multiple types of solid tumors yet failed to yield any success to date in GBM [5]. Multiple phase III clinical trials on programmed cell death protein 1 (PD-1) inhibitor nivolumab have been conducted but did not show any benefit in OS in both recurrent[27] and newly-diagnosed GBM (NCT02617589; NCT02667587). Interestingly, before neurosurgical resection, neoadjuvant administration of anti-PD-1 pembrolizumab showed an enhanced local and systemic anti-tumor immune response in recurrent GBM, which warrants further investigation [28]. These findings show that immune checkpoint inhibitors (ICIs) can have potential in the treatment of GBM but optimal timing of using ICIs warrants further investigation.

Since tumor neoantigens are needed to invoke an anti-tumor immune response, observations that GBM exhibits relatively low mutational and predicted neoantigen burden hampers the efficacy of immunotherapy [29]. Furthermore, GBM is characterised by a relatively low influx of effector T-cells, limiting ICI efficacy [30]. Efforts are mainly being made to overcome intrinsic and/or adaptive resistance and immunosuppression by using chimeric antigen receptor (CAR)T cells (i.e., targeting EGFRvIII; NCT03726515) dendritic cell vaccines [31].

All in all, none of the conducted clinical trials have led to a vast improvement in GBM patient outcomes. Several factors have been attributed to this lack of success, including poor BBB penetration of the drug, lack of adequate patient stratification in trial designs, the development of treatment resistance, and, most importantly, the extensive tumor heterogeneity in GBM [5].

Tumor Heterogeneity

Tumor heterogeneity and cancer stem cells

Decades of cancer research have established that cancer cannot be seen as simply one disease but comprises a wide range of different subtypes, even within tumors that develop from the same tissue. This includes inter-tumor heterogeneity (tumor characteristics differ between patients) and intratumoral heterogeneity (tumor characteristics differ within one patient). These concepts explain why not all patients with the same tumor type respond in the same way to one treatment and why tumors sometimes only partially respond, eventually recur, and become resistant to therapy.

Intratumoral heterogeneity has been explained by two proposed models [32]. First, the clonal evolution model is based on natural selection in which stochastic mutations in individual tumor cells occur, leading to different subclones. This results in adaptation and selection for the fittest clones of a tumor by acquiring growth advantage based on requirements present in different tumour areas and selection under the pressure of treatment [33]. The second model introduces the concept of cancer stem cells (CSCs). CSCs are defined as a subset of cancer cells that possess the ability for indefinite self-renewal and tumor initiation and growth. This model proposes that tumors are hierarchical and introduce intratumoral heterogeneity by installing a differentiation hierarchy [34]. Importantly, each model has different therapeutic implications: the CSC model requires the eradication of CSC only while the clonal evolution model requires killing all cells to achieve cure.

However, it is important to note that both models are likely to exist in human cancer as CSCs have also been shown to undergo clonal selection [35]. Also, cancer cells are plastic as terminally differentiated cells can also gain CSC properties like self-renewal due to novel mutations and micro-environmental influences. This can subsequently lead to the establishment of a new hierarchical CSC clone [32]. Thus, every cancer must be seen and treated as a unique disease. Consequently, indivdualized spatial interrogation of patient tumors is needed to tailor successful treatment.

In GBM, the existence of so-called glioma stem cells (GSCs) has also been proposed. $CD133^+$ cells were the first to be described to exhibit stem cell properties *in vitro* and also showed tumor-initiating properties in a mouse xenograft model, whereas $CD133^-$ cells could not form tumors [36]. This was, however, later disputed as a subset of $CD133^-$ cells were also identified to have tumor-initiating properties [37]. Since multiple other markers for GSCs have been proposed, including stage-specific embryonic antigen-1 (SSEA-1) [38], Nestin [39], oligodendrocyte transcription factor 2 (OLIG2) [40], sex-determining region 2 (SOX2) [41], and CD44 [42]. GSC markers, however, remain controversial as no marker or set of markers has been identified that exclusively and comprehensively mark GSCs [43,44].

Glioblastoma tumor heterogeneity

Genomic analysis of a large cohort of primary GBM samples identified several significantly mutated genes in GBM. These include phosphatase and tensin homolog (PTEN), tumor protein 53 (TP53), EGFR, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA), phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1), neurofibromin 1 (NF1), retinoblastoma 1 (RB1), IDH1 and PDGFRA [45]. Other main genetic alterations found in GBM include amplification events on chromosome 7 (EGFR/MET/CDK6), chromosome 12 (CDK4 and mouse double minute 2 homolog (MDM2)), and chromosome 4 (PDGFRA), as well as homozygous deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A) [45].

Bulk RNA-sequencing identified gene expression-based molecular subtypes: proneural, classical, mesenchymal, and neural [46]. These subtypes could roughly be linked to genetic alterations. Proneural GBM was characterised by alterations of PDGFRA and TP53 and point mutations in IDH1. Classical GBM was associated with EGFR amplification, homozygous deletion of CDKN2A, and lack of TP53 mutations. The mesenchymal subtype included genetic alterations in NF1 and PTEN. Finally, the neural subtype showed expression of specific neuron markers but is less well characterised [46]. Notably, single cell RNA-sequencing has shown that these GBM subtypes vary across individual cells within a tumor [47]. This molecular heterogeneity was also confirmed by multisampling of spatially distinct tumor fragments [48]. Additional single cell RNA-sequencing data identified different cellular states in which GBM cells can exist. It was shown that GBM cells can exist in neural progenitor-like (NPC-like), oligodendrocyte progenitor-like (OPC-like), astrocyte-like (AC-like) and mesenchymal like (MES-like) states [49]. Genetic alterations also characterise these states. GBMs dominant with AC-like cells were enriched for EGFR alterations, similar to the classical subtypes, whereas GBMs dominant with MES-like cells correlated with NF1 mutations, similar to the mesenchymal subtype. OPC-like and NPC-like GBMs correlated with the proneural subtype and were associated with high-level amplifications of PDGFRA and CDK4 in OPC-like and NPC-like GBMs respectively [49].

Additionally, these cell states were shown to co-exist within one tumor in different ratios. Importantly, it was also shown that GBM cells are highly plastic and can change their state during tumor evolution [49]. This data highlights the tumor cell heterogeneity within GBM and the adaptive properties these cells possess, complicating effective treatment of GBM.

Adding another layer of complexity to the extensive heterogeneity in GBM are the temporal changes that occur during tumor evolution and under treatment pressure. For example, genomic and transcriptomic analysis showed that 63% of the 114 patients studied experienced expression-based subtype changes at tumor recurrence after treatment [50]. Also, a subset of TMZ-treated patients (17 out of 100) relapsed with hypermutated tumors, probably related to acquired mismatch repair (MMR) deficiencies [50]. These findings emphasise that GBM tumors can significantly change between primary and recurrence, so determining treatment options for recurrent tumors based on data on the primary tumor is problematic and should be done with caution.

Apart from tumor cell heterogeneity, the TME also plays an essential role in intratumoral heterogeneity and treatment resistance [51]. GBM is characterised by extensive areas of hypoxia, which are associated with a more aggressive tumor phenotype [52], the creation of an immunosuppressive microenvironment and a well-known contributor to radio- and chemoresistance[53]. In addition, intratumoral hypoxia contributes to intratumoral heterogeneity by maintaining GSCs [52], inducing metabolic changes [54] and phenotypic adaptations in GBM cells which are also plastic under the influence of dynamic changes in hypoxia [55].

Another significant influence from the TME is the immune micro-environment. GBM is generally seen as an immunogenic *cold* tumor, characterised by a relatively low

number of tumor neoantigens, little infiltration of effector immune cells within the tumor and a vast immunosuppressive environment.

The majority of GBM only have small numbers of tumor-infiltrating lymphocytes (TILs) [30]. The TILs that do infiltrate the TME often exhibit an exhausted phenotype [56] even after immunogenic stimulation by vaccines [57].

In GBM, tumor-associated macrophages (TAMs) can make up to 30% of the tumor mass and, therefore, the most dominant immune cell subtype present in TME [30]. Unique to the brain TME is the co-existence of two distinct macrophage lineages. Microglia are brain-resident macrophages that develop from embryonic yolk sac progenitors and are independent from peripheral hematopoiesis. During GBM development, and due to disruption of the BBB, bone-marrow derived macrophages (BMDMs) also enter the TME [30]. However, whether these different types of macrophages have distinct functions in the brain TME is still controversial [30]. These TAMs can be programmed towards a pro-inflammatory M1 phenotype as well as an anti-inflammatory M2 phenotype under the influence of environmental cues [58]. However, it has been shown that macrophages are plastic in their phenotype and can also exist in intermediary states, adding another level of complexity in intratumoral heterogeneity [59].

Multiple factors contribute to the creation of an immunosuppressive environment in GBM [60]. Astrocytes produce immunosuppressive cytokines interleukin-10 (IL-10) and transforming growth factor beta (TGFβ) in response to inflammatory stimuli derived from tumors. TGFβ is also involved in programming TAMs towards an anti-inflammatory phenotype, which also produces IL-10 and TGFβ, further enhancing immunosuppression [60]. TAMs also produce arginase which depletes arginine levels and as a result inhibits T cell proliferation and function [61]. GBM cells produce indolamine 2,3-dioxygenase (IDO), which stimulates the accumulation of regulatory T-cells (Treg) through tryptophan depletion and suppresses T-cell function [62].

To sum up, the existence of intratumoral heterogeneity – both at a molecular, cellular and micro-environmental level, greatly complicates durable responses in GBM patients. Unfortunately, at this moment, tumor heterogeneity is not accounted for in daily clinical practice and proper methods to evaluate changes in tumor heterogeneity during tumor evolution and recurrence are lacking. Therefore, future treatment of GBM should be done using a personalised approach, in which individual intratumoral heterogeneity is accounted for both on a preclinical and clinical research level.

Dealing with tumor heterogeneity in preclinical and translational research

Traditional preclinical and translational research uses (immortalised) cancer cell lines that are accessible to culture and manipulate *in vitro* but lack a genetic and phenotypic resemblance of tumors and intratumoral heterogeneity [63]. In the past decade, patient-derived cancer organoids have been developed and widely used *in vitro* models to study cancer biology and identify novel treatment options. In 2015 the first organoid biobank, derived from colorectal cancer, was developed [64] and since, patient-derived cancer organoids have been established for a wide range of solid tumors [65].

Patient-derived cancer organoids are three-dimensional stem-cell derived cultures that resemble the original tumour's phenotypic, genetic and transcriptomic characteristics. Due to the stem-cell nature of these cultures, organoids retain the capacity of self-renewal and the ability to undergo multilineage differentiation [66]. Importantly, single-cell analysis of these organoids have shown that cancer organoids retain intratumoral heterogeneity and tumor clonal hierarchy [67,68]. Furthermore, cancer organoids develop central regions of hypoxia and necrosis; major determinants in chemo- and radioresistance that do not occur in 2D cell culture [69]. More recently methods have been developed to establish multi-cell type cancer organoids by including fibroblasts, endothelial cells, neuronal cells and immune cells [70-72]. Additionally, cancer organoids are less expensive and labor-intensive when compared to (patient-derived) xenograft models which also reduces laboratory animal usage. Therefore, patient-derived cancer organoids are increasingly used in cancer research as they more closely resemble the *in vivo* tumor.

Essential aspects of organoid culture include determining optimal growth conditions and establishing how cancer organoids resemble the parental tumor. A large-scale cancer organoid platform from more than 1000 patients has been established, showing the potential of establishing cancer organoids from multiple types of solid tumors with success rates varying from 15 to 75% depending on cancer subtype [73]. Using this approach, this study was able to determine minimal growth factor dependence for organoid growth initiation and establish the genetic and transcriptomic concordance between cancer organoid and parental tumor [73].

Overall, there is a large increase in the use of cancer organoids in cancer research. In preclinical and translational research, cancer organoids have been used to study cancer mutational signatures [74] and consequences of specific mutations on tumorigenesis or

treatment response using genetic modifications [75]. Additionally, from a more clinical point of view, cancer organoids have been used to study treatment response, including both *in vitro* high-throughput drug screens [73]as well as using organoids as a predictive platform for individualised patient treatment selection [76].

Appraising tumor heterogeneity in the clinical setting

In the clinic, histopathological and molecular analyses of the resected tumor provide information on the intratumoral heterogeneity within GBM. However, currently used methods cannot ascertain heterogeneity on the single-cell level and implementing methods that are expensive and not universally available. It should also be taken into account that not all GBMs are eligible for complete tumor resection. For example, due to an eloquent tumor location, only a single biopsy can be taken for diagnostic confirmation, which can dramatically underestimate the extent of heterogeneity. Additionally, GBM is highly infiltrative in the healthy brain tissue, so an actual complete tumor resection is never achieved. Tissue collection at recurrence is often even more complex and monitoring the dynamic changes during treatment through continuous resampling is impossible to implement. Due to these reasons, noninvasive diagnostic methods are investigated to determine tumor heterogeneity and monitor tumor evolution.

Radiological approaches using advanced MRI techniques and artificial intelligence (AI) offer a possible method to achieve this. Qualitative analysis of conventional MRI using the Visually AccesSAble Rembrandt Images (VASARI) features has shown to be reproducible and potentially predictive for OS [77] and molecular subtype [78].

More recently, computational AI approaches for the quantitative analysis of medical images have gained more attention within cancer research. For example, radiomics has been proposed to extract imaging features by high-throughput data mining on textures, shapes, and intensities [79] and has shown prognostic and predictive value in multiple solid tumors [80,81].

The first studies on radio(geno)mics focused on computed tomography (CT) imaging. However, as MRI is far superior for brain imaging and is the primary modality used in clinical practice for GBM, newer studies have investigated MRI radiomics analysis. Whereas CT images can all be quantified using standardised Hounsfield units, no such unit exists in MRI imaging. This provides challenges as preprocessing steps have

to be conducted in order to try to account for differences in MRI acquisition protocols between different centers. These preprocessing steps are still under investigation as not all radiomics features were robust between different preprocessing methods [82].

In GBM, MRI radiomics has been used to predict patient survival [83], molecular tumor alterations [84], and other biological aspects such as tumor grade [85] or pseudoprogression [86]. However, it is important to note that most studies published to date only use single-institute data, lacking inter-hospital variation and only use internal validation methods, and lack external validation, all essential steps in establishing clinically relevant and universally applicable radiomics signatures.

Another method of noninvasive diagnostics is the usage of liquid biopsies. Analysis of peripheral blood samples has shown that circulating tumor cells (CTCs) are scarce in GBM patients [87]. However, that circulating tumor DNA (ctDNA) is much more abundant, though still less when compared to other solid tumors [88]. Thus, for GBM patients, ctDNA extraction from cerebrospinal fluid CSF) might be more effective as ctDNA is more abundant in the CSF, and parallel sequencing has shown that ctDNA from CSF is more effective and comprehensive to catalog the genomic alterations of brain tumors when compared to plasma [89]. Further research includes analysis for circulating cell-free tumor RNA (ctRNA), extracellular vesicles (EVs), circulating proteins and metabolites, and tumor-educated platelets as possible prognostic or predictive markers in GBM [90].

Outline of the thesis

This thesis aimed to identify novel methods to study tumor heterogeneity in GBM and integrate tumor heterogeneity in future translational and clinical studies.

In **Chapter 2,** we review the potential utility of patient-derived cancer organoids as predictors for treatment response. We highlight current studies that have directly compared treatment response in organoids and matched patient response in the clinic. Additionally, we discuss the current limitations and opportunities within the field of cancer organoids.

In **Chapter 3,** we show the development and validation of a patient-derived GBM organoid model. This study shows the phenotypic and genetic heterogeneity is largely maintained within these GBM organoids and the resemblance toward the

parental tumor. Furthermore, we show the potential of GBM organoids to test new treatment options and identify novel treatment resistance mechanisms.

Chapter 4 reviews the available noninvasive diagnostic methods to account for tumor heterogeneity in clinical practice. We discuss conventional radiological approaches, as well as advanced computational approaches and liquid biopsies.

In **Chapter 5,** we develop a prognostic and predictive model based on qualitative and quantitative MRI analysis. This study shows the additive value of MRI analysis upon clinical variables alone to predict OS in GBM patients. Additionally, we explore the potential of integrated MRI analysis to predict clinically relevant molecular markers and discuss current limitations that need to be faced before the actual clinical implementation of such models can be achieved.

In **Chapter 6,** we use high-dimensional multiplex immunohistochemical analysis of a GBM patient cohort to investigate the spatial tissue architecture of GBM tumor cells and their interaction with the genetic background and spatial immune micro-environment. Also, we discuss the potential clinical implications of these findings regarding patient stratification for novel treatment options.

In **Chapter 7,** the findings of the thesis are summarised, followed by a general discussion and appraisal of future perspectives and clinical implications.

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Chapter 2

Patient-derived cancer organoids as predictors of treatment response

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Abstract

Patient-derived cancer organoids have taken a prominent role in pre-clinical and translational research and have been generated for most common solid tumors. Cancer organoids have been shown to retain key genetic and phenotypic characteristics of their tissue of origin, tumor subtype and maintain intratumoral heterogeneity and therefore have the potential to be used as predictors for individualized treatment response. In this review, we highlight studies that have used cancer organoids to compare the efficacy of standard-of-care and targeted combination treatments with clinical patient response. Furthermore, we review studies using cancer organoids to identify new anti-cancer treatments using drug screening. Finally, we discuss the current limitations and improvements needed to understand the full potential of cancer organoids as avatars for clinical management of cancer therapy.

Introduction

Cancer remains one of the leading causes of death in the 21st century. Despite enormous progress in the identification of mechanisms of tumor progression and treatment resistance and the development of new tumor targeted treatments many patients do not get cured. The main challenge remains to achieve accurate patient selection. Tumor biopsies used for clinical diagnostics do not capture the extensive intratumoral heterogeneity that masks emergent tumor clones with intrinsic or acquired resistance. This can result in patients receiving suboptimal treatment, or overtreatment leading to long-lasting harmful side-effects. The development of specific biomarkers and predictive model systems are therefore essential to personalized treatment leading to more durable responses with less side effects.

In the last decade, translational cancer research has witnessed a revolution with the development of methods that enable the reproducible derivation, maintenance and biobanking of primary human normal and cancer tissues. These primary cell cultures, called organoids, are three dimensional stem-cell derived cultures that support the propagation of phenotypic, genetic and transcriptomic characteristics from the original tissue and retain the self-renewal properties of stem cells and their ability to undergo multilineage differentiation indefinitely. This revolution took off after the development of 'mini-guts' from Lgr5+ intestinal stem cells which replicate the dynamic proliferation and differentiation of the intestinal crypt epithelium in culture (Sato et al., 2009) and thereafter from colorectal cancer biopsies to derive colorectal cancer (CRC) organoids (van de Wetering et al., 2015). Importantly cancer organoids have been shown to retain intratumoral heterogeneity and tumor clonal hierarchy; a major limiting factor in treatment effectiveness and recurrence (Roerink et al., 2018). Excellent comprehensive reviews on the derivation and characterization of organoid systems can be found here and will not be further elaborated on in this review (Clevers, 2016). To date cancer organoids have been developed from many cancer types and have been shown to maintain a stable genetic and phenotypic representation of the original tumor in culture compared to classic 2D monolayer cell cultures. Like patient-derived xenograft (PDX), cancer organoids also main intratumor heterogeneity but are less expensive and labor-intensive and reduce laboratory animal usage. On the other hand, PDX models include the tumor micro-environment and in vivo drug metabolism which are currently not accounted for in cancer organoids (Weeber et al., 2017).

Whether cancer organoids can also function as avatars for prospective target identification and treatment selection at the patient level is not yet known. If so,

cancer organoids could have a profound impact on individualized cancer treatment. In this review, we describe studies that have addressed whether cancer organoids are predictors for clinical response.

Materials & Methods

A literature search was conducted using publicly available databases (PubMed, MEDLINE, and Web of Science) up to October 2020. For the purpose of this review, cancer organoids were defined as stem-cell derived three-dimensional cell culture models derived from primary patient-derived solid tumors using a basement membrane extract (BME). Articles describing patient-derived-xenograft (PDX)-derived cancer organoids, iPSC based models or three-dimensional cell culture systems lacking a BME (i.e. slice cultures or tumor-on-a-chip approaches) were excluded. Search keywords included [ORGANOID] or [TUMOROID] and, [CANCER] and [TREATMENT]. Only articles in which cancer organoids were subjected to treatment and correlated to patient outcome or potential biomarkers were included. Establishment, characterization and comparison of cancer organoids towards the parental tumor have already been previously described and fall outside the scope of this review (Drost and Clevers, 2018;Bleijs et al., 2019).

Results

A total of 60 studies were included in this review. For almost all papers the cancer organoids used were (previously) compared on a genetic and phenotypic level to the parental tumor in order to validate the model. Study characteristics are summarized in Table 1.

Gastro-intestinal (GI) cancer

In 2015 the first colorectal cancer (CRC) organoid biobank was established and used for drug screening. Based on these drug screens, gene-drug interactions could be studied to identify potential biomarkers and study the molecular basis for drug response to both new therapeutic agents as well as current standard-of-care chemotherapeutics (van de Wetering et al., 2015).

Esophageal adenocarcinoma organoids were subjected to standard-of-care chemotherapy (5-FU, epirubicin and cisplatin). All but one of the patients used for organoid derivation showed poor clinical response to chemotherapy which was

recapitulated in the corresponding cancer organoids, though organoids from the patient that showed clinical response were not available for drug testing (Li et al., 2018). This overlap between organoid and tumor response for four patients towards chemotherapeutics (cisplatin, paclitaxel, 5-FU, epirubicin and irinotecan) was also observed in another study (Derouet et al., 2020).

A similar approach was taken using gastric cancer organoids. In one study, gastric cancer organoids derived from one patient at pre-treatment were sensitive to standard-of-care chemotherapy (5-fluorouracil (5-FU), cisplatin, oxaliplatin and irinotecan) which reflected the complete pathologic response in the patient after chemoradiation, albeit the contribution of radiotherapy to the clinical response was not investigated (Gao et al., 2018). In another study conflicting results for combined treatment with 5-FU, oxaliplatin and epirubicin were obtained. From the seven gastric cancer patients included in the study, correlation of treatment sensitivity with clinical response could only be made for two patients. Only for one of these patients the organoid response matched the clinical response (Steele et al., 2019).

Ascites-derived gastric cancer organoids showed a heterogeneous response towards standard-of-care chemotherapeutics between patients, similar to the mixed clinical responses seen in patients with peritoneal metastases, though no direct clinical comparison could be made (Li et al., 2019a).

CRC organoids were used to identify patients that benefit from the PARP inhibitor olaparib and cross sensitivity to oxaliplatin, which causes PARP-dependent DNA damage repair. In two patients that responded to oxaliplatin the organoids were sensitive to both olaparib and oxaliplatin. In another patient that responded, the organoids showed resistance to both treatments. Notably the organoids from this patient were highly responsive towards panitumumab which was also part of the clinical treatment and might have been a main factor in the clinical response and explain the discrepancy between organoid and clinical response (Arena et al., 2020).

The TUMOROID study derived organoids from metastatic CRC and found organoids to be predictive of response to irinotecan-based therapies (predicted response in more than 80% of patients without misclassifying patients who would have benefited from treatment), but not predictive for 5-FU and oxaliplatin combined treatment. A possible explanation for this could be that the tumor micro-environment (stromal and immune cells), not present in organoids, might influence the efficacy of one treatment more than the other (Ooft et al., 2019). In another study, organoids from one metastatic CRC patient showed an intermediate response towards 5-FU and

Table 1: Summary of study characteristics and main results

Study	Cancer type	Organoid establishment success rate (%)	# of organoid lines used for treatment experiments	Multiple organoid lines per patient	Matched healthy tissue organoids	Standard-of-care testing	Clinical compariosn	Drug screen	Outcomes regarding prediction of clinical treatment response
Gastro-intestinal cancer									
van de Wetering M. 2015	Colorectal cancer	90%	19	■	■	■	■	■	Drug screen on organoids. No clinical comparison.
Koppens M. 2016	Colon cancer	not reported	2	■	■	■	■	■	Efficacy of EZH2 inhibition in organoids. No clinical comparison.
Verissimo CS. 2016	RAS mutant colorectal cancer	n.a.	2	■	■	■	■	■	Organoid response towards EGFR-RAS-ERK targeting in relation to KRAS mutation status. No clinical comparison.
Buzzelli JN. 2018	Colorectal cancer liver metastases	76.5%	3	■	■	■	■	■	Efficacy of standard-of-care chemotherapy in organoids. No clinical comparison.
Vlachogiannis G. 2018	Metastatic gastrointestinal tumors	not reported	21	■	■	■	■	■	Sensitivity 100%, specificity 93%, PPV 88%, NPV 100% for organoids in forecasting clinical response to targeted agents or chemotherapeutics.
Gao M. 2018	Gastric cancer	not reported	2 (from 1 patient)	■	■	■	■	■	Testing of standard-of-care chemotherapeutics. Descriptive clinical comparison (N=1) showed lowest IC50 value for 5-FU (out of 4 chemotherapeutics tested) and clinical complete response after 5-FU/RTx treatment. No testing for contribution of RTx to clinical effect.
Li X. 2018	Esophageal adenocarcinoma	31%	9	■	■	■	■	■	Drug screen on organoids. Descriptive comparison showing lack of chemotherapy sensitivity in most organoid cultures which resembled the poor clinical response observed.
Yan HHN. 2018	Gastric cancer	50% (cancer), 100% (healthy)	9 (from 7 patients)	■	■	■	■	■	Drug screen on organoids. Descriptive comparison showing lack of organoid response to 5-FU in a patient that showed progressive disease upon 5-FU. Two other patients showed a clinical response to 5-FU/cisplatin which was resembled in the organoids.
Votanopoulos KI. 2019	Appendiceal cancer	75%	9	■	■	■	■	■	Chemosensitivity testing of organoids. No clinical comparison.
Li J. 2019	Gastric cancer (ascites-derived)	92%	7	■	■	■	■	■	Drug screening of organoids. No clinical comparison.
Schumacher D. 2019	Colorectal cancer	not reported	38	■	■	■	■	■	Efficacy of EGFR-targeted therapy in relation to KRAS mutation status in organoids. No clinical comparison.
Seidlitz T. 2019	Gastric cancer	not reported	4	■	■	■	■	■	Drug testing of organoids. No clinical comparison.
Ubink I. 2019	Colorectal peritoneal metastases	Not reported	5	■	■	■	■	■	Sensitivity to HIPEC chemotherapy and efficacy of addition of ATR inhibitor. No clinical comparison.
Pasch CA. 2019	Multiple types of cancer (treatment only on (m)CRC)	76%	5	■	■	■	■	■	Descriptive clinical comparison (N=1). Clinical response to FOLFOX in a patient of which the organoid showed an intermediate response towards 5-FU/oxaliplatin treatment.

Study	Cancer type	Organoid establishment success rate (%)	# of organoid lines used for treatment experiments	Multiple organoid lines per patient	Matched healthy tissue organoids	Standard-of-care testing	Clinical compariosn	Drug screen	Outcomes regarding prediction of clinical treatment response
Steele NG. 2019	Gastric cancer	not reported	6						Drug screening of organoids. Descriptive clinical comparison (N=2) showing a similar response in the organoid for one patient but not in the other.
Ganesh K. 2019	Rectal cancer	77%	21						Drug screening of organoids. Clinical comparison for chemotherapy (N=7) showing a correlation of AUC for both 5-FU and FOLFOX with progression-free survival of the corresponding patient (r=0.86, p=0.024). Descriptive comparison of radiosensitivity (N=7) showing organoid responds corresponds with clinical radiotherapy response.
Ooft SN. 2019	Mestastatic colorectal cancer	63%	Varies per treatment						Prediction of response to irinotecan monotherapy (N=10): accuracy of classifier 80%. Prediction of response to 5-FU/ irinotecan combination therapy (N=12): 83.3% correctly classified. Prediction of response to 5-FU-oxaliplatin (N=16): no correlation with clinical response.
Costales-Carrera A. 2019	Colon cancer	not reported	3						Efficacy of plocabulin in organoids. No clinical comparison.
Yao Y. 2020	Locally advanced rectal cancer	85.7%	80						High correlation between organoid response and clinical outcomes for prediction of neoadjuvant chemoradiation efficacy: AUC 88.20% (76.46-98.67%), accuracy 84.43% (72.40-93.75%), sensitivity 78.01% (55.56-95%), specificity 91.97% (77.78-100%).
Narasimhan V. 2020	Colorectal peritoneal metastases	68%	15						Drug screening of organoids. Descriptive clinical comparison (N=3) in which drug treatment was selected based on organoid sensitivity which was successful for 1 patient.
Zerp SF. 2020	Colorectal cancer	not reported	3						Efficacy of APG-880 as a radiosensitizer in organoids. No clinical comparison.
Derouet MF. 2020	Esophageal adenocarcinoma	57.2%	16						Descriptive clinical comparison (N=4) showing an overlap between the organoid and tumor response.
Arena S. 2020	Colorectal cancer	not reported	5						Drug testing on organoids. Descriptive clinical comparison (N=3) which corresponded with organoid sensitivity.
Abdominal (non-GI tract) cancer									
Huang L. 2015	Pancreatic cancer	not reported	5						Drug screen of organoids. No clinical comparison.
Broutier L. 2017	Liver cancer	44%	6						Drug sensitivity testing of organoids. No clinical comparison.
Nuciforo S. 2018	Hepatocellular carcinoma	26%	12						Efficacy of sorafenib on organoids. No clinical comparison.
Tiriac ML. 2018	Pancreatic cancer	75%	66						Descriptive comparison of organoid response towards clinical response. For one patient retrospective clinical data paralleled the chemosensitivity profile of the organoid.

Study	Cancer type	Organoid establishment success rate (%)	# of organoid lines used for treatment experiments	Multiple organoid lines per patient	Matched healthy tissue organoids	Standard-of-care testing	Clinical compariosn	Drug screen	Outcomes regarding prediction of clinical treatment response
Li L. 2019	Liver cancer	not reported	27 (from 5 patients)						Drug screen of organoids. No clinical comparison.
Hennig A. 2019	Pancreatic cancer	71%	10						Efficacy of standard-of-care chemotherapy stratified for KRT81 status. No clinical comparison.
Bian B. 2019	Pancreatic cancer	not reported	24						Efficacy of BET-inhibitor treatment on organoids. No clinical comparison.
Driehuis E. 2019	Pancreatic cancer	62%	24						Drug screen on organoids. Descriptive comparison towards clinical response (N=4) showing an overall correlation between organoid and clinical response.
Ponz-Sarvise M. 2019	Pancreatic cancer	not reported	2						Drug sensitivity testing of organoids. No clinical comparison.
Castven D. 2019	Liver cancer	11%	5						Testing efficacy of targeted agents based on mutational variants in organoids. No clinical comparison.
Sharick JT. 2020	Pancreatic and Breast cancer	64% (for pancreatic cancer), 54% (for breast cancer)	7 (pancreas), 11 (breast)						Using metabolic heterogeneity to predict treatment response in pancreatic cancer organoids (N=7). Three patients were classified as predicted non-responders and all showed tumor recurrence within one year whereas four patients that were classified as predicted responders all remained free of tumor recurrence for more than one year.
Seppälä TT. 2020	Pancreatic cancer	77%	13						Pharmacotyping of organoids. No clinical comparison.
Saltsman J. 2020	Hepatoblastoma	not reported	1						Drug testing on normal liver and tumor organoid from one patient. No clinical comparison.
Liu J. 2020	Liver cancer	not reported	4						Effect of co-culture system with cancer-associated fibroblasts on drug sensitivity in organoids. No clinical comparison.
Urogenital and gynaecological cancer									
Gao D. 2014	Metastatic prostate cancer or CTCs	15-20%	6						Sensitivity to androgenreceptor and PI3K inhibitors in organoids. No clinical comparison.
Girda E. 2017	Endometrial cancer	100%	14 (varies per drug)						Drug testing on organoids. No clinical comparison.
Lee SH. 2018	Bladder cancer	70%	11						Drug screen of organoids and comparison to in vivo (mice) response. No clinical comparison.
Puca L. 2018	Prostate cancer	16%	6						Drug screening on organoids. No clinical comparison.
Kopper O. 2019	Ovarian cancer	65%	21						Descriptive clinical comparison: organoids derived from clinical resistant recurrent disease were more resistant compared to the clinically sensitive primary disease counterpart (N=1). Drug screen of organoids and comparison to in vivo (mice) response.
Boretto M. 2019	Endometrial cancer	20%	5						Drug response to standard-of-care chemotherapeutics. No clinical comparison.

Study	Cancer type	Organoid establishment success rate (%)	# of organoid lines used for treatment experiments	Multiple organoid lines per patient	Matched healthy tissue organoids		Standard-of-care testing	Clinical compariosn	Drug screen	Outcomes regarding prediction of clinical treatment response
Mullenders J .2019	Bladder cancer	57.9%	3							Drug response to standard-of-care chemotherapeutics. No clinical comparison.
Calandrini C. 2020	Childhood kidney cancer	100% for healthy tissue, 75% for Wilms tumor, 100% for MRTK, 75% for RCC. Unsuccesfull for rare kidney tumor types	4							Drug screen of cancer and healthy tissue organoids. No clinical comparison.
de Witte C.J. 2020	Ovarian cancer	not reported	36							Drug screening on organoids. Organoid drug response to carboplatin+paclitaxel treatment showed significant correlation with clinical response (N=7, P<0.01). PDOs generated at interval debulking recapitulated the clinical response to first-line carboplatin and paclitaxel combination treatment for histopathological (p = 5.821e 05), biochemical (p = 0.0004), and radiological (p = 0.0092) outcomes.
Central nervous system cancer										
Hubert CG. 2016	Glioblastoma	not reported	1							Identification of radioresistant cells in organoids. No clinical comparison.
Saengwimol D. 2018	Retinoblastoma	83%	1							Effects of standard-of-care chemotherapeutics. No clinical comparison.
Scognamiglio G. 2019	Chordoma	not reported	3							Efficacy study of nivolumab. No clinical comparison.
Loong HF. 2020	Glioblastoma	n.a.	1							Prospective identification of everolimus as treatment option using organoids showing subsequent partial clinical response.
Chadwick M. 2020	Glioblastoma	not reported	4							Drug screen on organoids. No clinical comparison.
Breast cancer										
Sachs N. 2018	Breast cancer	>80%	28							Drug screening of organoids and comparison to in vivo response in mice. No clinical comparison.
Li X. 2020	Breast cancer	n.a.	1							Case-report for drug screening on organoids. No clinical comparison.
Pulmonary cancer										
Sachs N. 2019	NSCLC	28%	4							Response to multiple chemotherapeutics and TKI's. No clinical comparison.
Kim M. 2019	Lung cancer	87%	5							Response to docetaxel, olaparib, erlotinib and crizotinib. No clinical comparison.
Chen J. 2020	NSCLC	not reported	7							Response to chemotherapeutics and targeted agents in organoids. No clinical comparison.

Study	Cancer type	Organoid establishment success rate (%)	# of organoid lines used for treatment experiments	Multiple organoid lines per patient	Matched healthy tissue organoids	Standard-of-care testing	Clinical compariosn	Drug screen	Outcomes regarding prediction of clinical treatment response
Li Z. 2020	NSCLC	80%	12						Drug screen on organoids. No clinical comparison.
Head-and-neck cancer									
Tanaka N. 2018	Head-and-neck cancer	37.2%	4						Response to cisplatin and docetaxel. No clinical comparison.
Driehuis E. 2019	HNSCC	65%	13						Descriptive comparison of response to radiotherapy (N=7). Organoid response for 6 patients was similar to the observed clinical response. Healthy organoids were not subjected to treatment.
Driehuis E. 2019	HNSCC	n.a.	8						Efficacy of EGFR-targeted photodynamic therapy. No clinical comparison.

oxliplatin combination which mimicked the clinical response observed after re-treatment with FOLFOX (Pasch et al., 2019).

Treatment sensitivity analysis on peritoneal CRC metastasis organoids could not separate patients with clinical partial response from those with progressive disease after FOLFOX. However, most of the patients in this study received pre-operative chemotherapy which may have led to selection of chemo-resistant subclones. Two patients did not receive oxaliplatin-based therapies and showed the highest sensitivity towards the treatment in vitro. Furthermore, two treatment-refractory patients were treated with a drug that was selected based on the drug sensitivity observed in their corresponding organoids. One patient received vandetanib (pan-tyrosine-kinase inhibitor) which strongly reduced organoid viability, however no clinical response was observed. For another patient, gemcitabine showed an initial partial clinical response but after two additional months of treatment, disease progression was again observed (Narasimhan et al., 2020). Peritoneal CRC-metastases organoids were also treated for efficacy towards mitomycin C and oxaliplatin (commonly used in HIPEC; intra-peritoneal chemotherapy treatment) and showed a general resistance, corresponding with the high recurrence rates observed in clinic (Ubink et al., 2019).

Organoids from metastatic GI cancers also predicted sensitivity towards cetuximab (anti-EGFR monoclonal antibody), and reflected clinical resistance in a patient who, based on molecular markers (EGFR amplification and KRAS wild-type), was expected to respond to the treatment (Vlachogiannis et al., 2018).

Rectal cancer organoids were exposed to standard-of-care chemotherapy (5-FU alone or FOLFOX (5-FU with leucovortin and oxaliplatin)) or radiotherapy (single dose, 0-8Gy). A high correlation (r=0.86) for 5-FU or FOLFOX was observed when compared to the progression-free survival (PFS) of the corresponding seven patients. For radiotherapy, organoids that showed resistance to radiotherapy were derived from previously irradiated tumors or from tumors that showed no to minimal clinical response. On the other hand, more radiosensitive organoids were derived from patients who had a minimal 50% reduction in tumor circumference endoscopically or a near-complete or a clinical complete response following radiotherapy (Ganesh et al., 2019). Additionally organoids (N=80) treated with neoadjuvant chemoradiation (5-FU and irinotecan) also reported promising predictive value for clinical response to neoadjuvant chemoradiation (sensitivity 78.01%, specificity 91.97%) (Yao et al., 2020).

As a proof-of-concept, the influence of KRAS mutation-mediated resistance to EGFR-targeted therapy using cetuximab was tested in rectal cancer organoids. In accordance with clinical trial data, KRAS-mutated organoids showed more resistance towards cetuximab compared to the KRAS wild-type organoids (Ganesh et al., 2019). These findings were also confirmed in an independent rectal cancer organoid biobank (Janakiraman et al., 2020) and for combined EGFR and MEK inhibition in CRC organoids (Verissimo et al., 2016).

Multiple studies have used organoids to screen for efficacious targeted agents based on genomic targetable variants present in the organoids. This approach was taken using gastric cancer organoid biobanks, CRC cancer organoids and in esophageal cancer organoids in which drug screening approaches identified patient subsets with potential vulnerability to new targeted agents (Koppens et al., 2016;Pauli et al., 2017;Li et al., 2018;Yan et al., 2018;Costales-Carrera et al., 2019;Schumacher et al., 2019;Seidlitz et al., 2019).

Finally, organoids were used to improve current treatments. CRC organoids showed an enhanced response to radiotherapy when organoids were simultaneously exposed to radiosensitizer APG-880 (Zerp et al., 2020). Peritoneal CRC-metastases organoids were used to optimize HIPEC treatment. Because mitomycin C (used in HIPEC) mainly induces interstrand crosslinks which activates ATR, the addition of ATR inhibitors to mitomycin C improved treatment efficacy on cancer organoids, identifying a potential new clinical strategy (Ubink et al., 2019).

Hepatobiliary tract and pancreatic cancer

Pancreatic cancer is one of the most lethal malignancies as it is often diagnosed in an advanced stage, has a high recurrence rate and only a minor survival benefit can be achieved with systemic therapy. This resistance to gemcitabine was also observed in pancreatic cancer organoids, though no clinical correlation could be made (Huang et al., 2015).

An organoid-derived pharmaco-transcriptomic signatures was developed to predict drug sensitivity to gemcitabine monotherapy but it did not predict response in patients receiving a combination treatment with other chemotherapeutics (Tiriac et al., 2018). Similar correlation between clinical response and gemcitabine sensitivity in pancreatic cancer organoids were found by others as well (Driehuis et al., 2019c). Another study also showed the feasibility of pharmacotyping pancreatic cancer organoids in a timely manner to guide postoperative chemotherapeutic selection (Seppala et al., 2020).

Pancreatic cancer organoid cultures derived from multiple different metastatic sites from the same patient showed a differential sensitivity towards 5-FU, but not towards the other chemotherapeutics tested. This suggests the existence of cancer subclones that differ between metastatic sites which are maintained in their respective cancer organoids (Tiriac et al., 2018).

Optical metabolic imaging (OMI) was used to assess treatment response in pancreatic cancer organoids and classify patients. Three patients were classified as predicted non-responders and all showed tumor recurrence within one year whereas four patients that were classified as predicted responders all remained free of tumor recurrence for more than one year (Sharick et al., 2020).

Novel approaches for pancreatic cancer organoids include the co-culture with cancer-associated fibroblasts (CAFs) and using matched pancreatic ductal organoids. CAFs were shown to increase treatment resistance which shows the importance of tumor micro-environmental aspects on treatment efficacy (Liu et al., 2020). Using matched pancreatic ductal organoids and pancreatic cancer organoids the lack of therapeutic response of dual MEK-AKT inhibition observed in a clinical phase II trial (Chung et al., 2017) was investigated. These findings recapitulated in human and murine organoids a tumor cell specific negative feedback loop causing upregulation of ERBB2, which was not observed in normal pancreatic ductal organoids. This provides a rationale for combining dual MEK-AKT inhibition with ERBB2 blockade with a high therapeutic ratio (Ponz-Sarvise et al., 2019).

Liver cancer organoids can be derived from liver cancer subtypes (hepatocellular carcinoma (HCC) cholangiocarcinoma (CC) or mixed type (HCC/CC)). Overall differential sensitivity was found between organoids from different patients to chemotherapies (gemcitabine) and targeted therapies (taselisib, AZD8931, SCH772984 and dasatanib or sorafenib)(Broutier et al., 2017;Nuciforo et al., 2018), though no clinical correlations could be made.

Liver cancer organoids (n=7) were also subjected to targeted therapies (KRAS, MET and KIT targeting). Sensitivity to these treatments however did not fully correlate with the presence of these driver mutations (Castven et al., 2019). In a more comprehensive study, liver cancer organoids from different tumor regions from the same patient were developed (n=5, 27 organoid lines) and tested for their sensitivity to conventional therapies and drug screening. Most interestingly, pan-effective drugs were identified that could uniformly kill many organoid lines whereas other drugs were only moderately sensitive in a few organoids from the same patient. This study highlights the intratumoral heterogeneity and differential sensitivity towards treatment within one patient and that a single patient organoid may not be sufficient to predict treatment outcome (Li et al., 2019b).

Cancer organoids were also derived from pediatric hepatoblastoma. For one patient drug testing was performed on the matched normal liver and tumor organoids. This screen identified one drug (JQ1) to have an increased efficacy on tumor organoids compared to normal organoids whereas standard-of-care cisplatin had not differential effect (Saltsman et al., 2020). Matched normal liver tissue organoids were also developed by others, providing opportunities to test normal tissue toxicity (Nuciforo et al., 2018).

Urogenital and gynecological cancer

Bladder cancer organoids were tested for sensitivity to chemotherapeutics (epirubicin, mitomycin C, gemcitabine, vincristine, doxorubicin or cisplatin), though no correlations could be made with patient response (Mullenders et al., 2019). Another study took a drug screening approach using bladder cancer organoids and observed strong, but variable responses. For example, in some organoids from patients with FGFR3 activating mutations, MEK/ERK inhibition was effective but not in all. Correlations were seen between more aggressive clinical phenotypes (metastasis and recurrence) and treatment resistance to a wide range of drugs in organoids (Lee et al., 2018).

A biobank (>50 organoid lines) of pediatric kidney cancer organoids was used to test treatment sensitivity towards standard-of-care chemotherapy (neoadjuvant actinomycin D (ACT-D) and vincristine; adjuvant doxorubicin and/or etoposide) on a specific subset of pediatric kidney cancers (Wilms tumor). Organoids derived from patients that received neoadjuvant chemotherapy were less sensitive to vincristine than those from patients not receiving prior chemotherapy. This suggests that resistance already develops in vivo and is maintained in cancer organoids. Furthermore, high-throughput drug screens in Wilms tumor organoids identified MEK and HDAC inhibitors as novel candidate interventions. Importantly, matched normal kidney organoids used in this study as well were equally sensitivity to romidepsin (HDAC inhibitor) and MEK inhibition compared to the tumor organoids. For pabinostat (pan-HDAC inhibitor) a significant increased sensitivity was observed in the tumor organoids compared to the normal kidney organoids making this the most interesting for clinical use. Using this approach, this study sets an example of using matched healthy and tumor organoids to identify treatments with the

best therapeutic ratio, considering both tumor efficacy and normal tissue toxicity (Calandrini et al., 2020).

Prostate cancer organoids were developed from neuro-endocrine prostate cancer (NEPC) and used for screening of cytotoxic drugs and identified alisertib (aurora A-kinase inhibitor). Organoids from two patients enrolled in a phase 2 clinical trial of alisertib mimicked the clinical response of the patients (one responder, one non-responder), supporting the potential clinical relevance of NEPC organoids as a predictive platform (Puca et al., 2018;Beltran et al., 2019).

Castrate-resistant prostate cancer (CRPC)-derived organoid lines (n=7) could successfully be established from biopsies and circulating tumor cells from all subtypes. A very limited drug study showed a response towards androgen suppression therapy (enzalutamide) in an organoid with androgen receptor amplification. This organoid line also showed a response to everolimus (mTOR inhibitor) and buparlisib (PI3K inhibitor) correlating with mutations in PTEN and PIK3R1 (Gao et al., 2014).

Endometrial cancer organoids (EC-O) representing early hyperplastic endometrium (n=13) and different stages and grades were derived (n=16) and exposed to standard-of-care treatment (paclitaxel, 5-FU, carboplatin, doxorubicin) and everolimus. These studies showed differential response between organoids from different patients (Boretto et al., 2019). Similar patient-specific responses were observed in another study with low- and high-grade EC-O (n=15) (Girda et al., 2017). Additionally, organoids from a patient with uterine carcinosarcoma and a patient with endometrial adenocarcinoma were used for drug screening. In both patients PI3K-inhibitors (buparlisib) were most effective, consistent with PIK3CA mutation present and strongly interacted with HDAC inhibitors as the most potent combinations treatment for both cancer organoids. (Pauli et al., 2017). For all three studies, no clinical comparison could be made.

Ovarian cancer organoids (OC-O) were successfully developed from multiple stages and subtypes (56 organoid lines). Standard-of-care drugs (platinum/taxanes) as well as targeted agents (PIK3K/AKT/mTOR inhibitors or PARP inhibitors) were used to test treatment sensitivity of OC-O. Unsupervised hierarchical clustering based upon platinum/taxane sensitivity could distinguish chemosensitive high-grade serous (HGS) organoids and chemoresistant non-HGS organoids which corresponded to the clinical findings. For one patient, HGS organoids clustered with the resistant group, corresponding to its clinically chemo-resistant phenotype and status as

recurrent disease. The pre-treated counterpart of this patient did cluster with the chemo-sensitive group, correlating with clinical behavior (Kopper et al., 2019). Another study also developed OC-O from different subtypes and found significant correlations between organoid response towards carboplatin and paclitaxel combination treatment and clinical response (N=7, $p<0.01$). During follow-up, organoid drug response did not correlate with 6-month progression-free survival. However, organoids derived from the patient with the shortest overall survival was least responsive. Interestingly, for a subset of patients, organoids were derived from multiple biopsies from the same tumor. This identified a differential response in monotreatment between different cancer lesions of seven patients in 31% of the cases, with at least one-mismatch for every drug tested. This emphasizes that intratumor heterogeneity may not be captured by a single biopsy indicating the importance of using multiple biopsies from different locations to predict sensitivity (de Witte et al., 2020).

Central nervous system cancer

As a proof-of-concept, organoids from one GBM patient, progressive after standard-of-care treatment, was used to identify potential drug candidates based on genomic alterations. This identified everolimus to be a potential therapeutic agent which correlated with a partial clinical response in this case-study (Loong et al., 2020). Cancer organoids derived from GBM patients were also used to test drug sensitivity to both standard-of-care chemotherapy (temozolomide) and molecular targeted agents towards mTOR, PI3K or DNA damage response. Differential response towards monotherapy as well as combined treatments with temozolomide and targeted agents was observed between organoids from different patients (Chadwick et al., 2020).

Chordoma organoids, a rare spinal cancer, were established that retained PD1 positive CD8 T-cells and were used to predict response towards nivolumab (PD-L1 blockade). A dose-dependent effect was observed in both PD-L1 positive and PD-L1 negative patients which corresponds with previous findings that low expression of PD-L1 can still lead to responses towards PD-L1 blockade (i.e. the approval of PD-L1 inhibitor pembrolizumab in NSCLC starting at 1% PD-L1 positivity). This study shows that this treatment response can potentially be predicted in cancer organoids, regardless of PD-L1 status though no correlation towards individual patient response could be made (Scognamiglio et al., 2019).

Multiple organoid lines of retinoblastoma were developed and tested for response to standard-of-care chemotherapy (melphalan, topotecan and methotrexate) which showed a similar response towards tumor cells in advanced disease in

clinical practice, but here no direct comparison to the patient response was made (Saengwimol et al., 2018).

Breast cancer

Breast cancer is one of the most prevalent types of cancer, comprising over 20 different subtypes. To include all these subtypes, a large (>100 organoid lines) breast cancer organoid biobank was established. For 12 patients with metastatic breast cancer a comparison with patient outcome was made. In this subset, the in vitro response to tamoxifen (estrogen receptor antagonist) matched that of the patients, showing their potential as treatment predictors. This biobank was used for high-throughput drug screening in which most, but not all, organoids responded to treatment as predicted from their mutations. Some organoid lines were insensitive to HER2 targeting despite HER2 overexpression which emphasizes the value of functional in vitro drug tests using cancer organoids (Sachs et al., 2018).

In another proof-of-concept study breast cancer organoids were derived from one patient and drug screening identified fulvestrant (estrogen receptor antagonist) as the most optimal treatment for this patient whereas based on genetic analysis (PTEN mutant), everolimus was expected to be the most effective treatment. Possibly this discrepancy can be explained by subclonal PTEN alterations resulting in differential efficacy of everolimus. Since this patient was not treated with either of these agents no correlation to the clinical response could be made, however it does show the additive value of drug screen on cancer organoids to genetic analysis of the tumor (Li et al., 2020a).

Pulmonary cancer

Lung cancer is the leading cause of cancer mortality and can be subdivided in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The first lung cancer organoid biobank was established using 80 lung cancer (SCLC and NSCLC) patients. Drug sensitivity testing was performed for both cytotoxic drugs (docetaxel) and targeted agents (olaparib and erlotinib). Sensitivity to PARP inhibitor olaparib correlated with BRCA2 mutation status as expected. EGFR-targeting by erlotinib did correlate with EGFR mutation status in most, but not all, lung cancer organoids. In one patient that harbored an EGFR mutation but was resistant to erlotinib, a MET amplification was present explaining resistance (Kim et al., 2019). In another study, NSCLC organoids were derived from lung adenocarcinoma using a TP53 activator (nutlin) to eliminate normal lung stem cells and organoids were used as a potential drug screening model from which findings could be correlated to molecular markers. Lung cancer organoids with an ALK1 mutation were shown to be resistant to crizotinib whereas ERBB2 mutated cancer organoids were sensitive to erlotinib and gefitinib (Sachs et al., 2019). Both studies did not compare the organoid response with that observed in the patient. Two more studies used NSCLC organoids for drug screening and correlation to molecular alterations found in the tumor. Both studies identified differential response towards treatment between organoids from different patients (Chen et al., 2020;Li et al., 2020b) but also some treatments (such as vincristine) which showed comparable activity across all organoid lines (Li et al., 2020b).

Head-and-neck cancer

Head-and-neck squamous cell carcinoma (HNSCC) organoids were developed and tested for treatment response towards standard-of-care chemotherapy in the metastasized setting (cisplatin and docetaxel). This study observed the highest IC50 value for docetaxel in the organoids derived from a pre-treated relapsed patient. Furthermore, in vitro resistance towards either one of the treatments could be confirmed in in vivo mouse models (Tanaka et al., 2018). HNSCC organoids were subjected to drug screening for targeted agents and standard-of-care treatments (cisplatin, carboplatin, cetuximab or radiotherapy) and differential responses were observed between organoids from different patients. Additionally, for a subset of patients, radiosensitivity of the organoids could be compared to clinical response. For six out of seven patients the organoid response towards radiation was similar to the clinical outcome of the patient (Driehuis et al., 2019a). Another study used HNSCC organoids to test a novel treatment approach, EGFR-targeted photodynamic therapy. A patient-specific response was observed which correlated with EGFR levels exhibited in the tumor and corresponding organoids (Driehuis et al., 2019b).

Discussion

In the past decade, cancer organoids have been established for large set of solid tumors and extensively characterized on a genetic, transcriptomic and phenotypic level. Overall, the conclusion is that cancer organoids are genetically and phenotypically stable replicates of the tissue and tumor subtype characteristics. Multiple studies have also used cancer organoids for drug screening approaches. However, only few studies have been able to make a quantitative clinical comparison to derive predictive values (Vlachogiannis et al., 2018;Ooft et al., 2019;de Witte et al., 2020;Yao et al., 2020). Most studies to date still provide a descriptive comparison between the organoid and clinical response which highlights the potential value of cancer organoids for this utility. Nonetheless, larger cancer organoid studies that make a quantitative comparison to the clinical response are warranted to make

accurate statements about the sensitivity and specificity of cancer organoids as clinical predictors of response and outcome. Cancer organoids have the potential to improve patient selection as multiple studies have shown that in some cases cancer organoids responded differently than predicted by driver mutations in the tumor (Sachs et al., 2018;de Witte et al., 2020). This could for example be due to small tumor subclones or mutations downstream or parallel to the targeted pathway (Kim et al., 2019). Recently, a protocol has been published as a standardized method to successfully establish organoids from different cancer types and perform drug screening thereof (Driehuis et al., 2020). Such guidelines are crucial to develop a robust and reproducible co-clinical platform for cancer organoids.

Limitations in cancer organoids studies

Several limitations currently exist that need to be addressed before cancer organoids can be implemented as a co-clinical track to aid clinical decision making. First, the derivation of organoids is not equally effective for all solid cancers. For example, NSCLC has shown a low establishment rate due to frequent overgrowth of lung cancer organoids by normal airway cells (Dijkstra et al., 2020). This overgrowth of somatic stem cells has been observed in the derivation of liver, prostate and endometrial cancer organoids as well (Gao et al., 2014;Broutier et al., 2017;Boretto et al., 2019). Approaches using omission of growth factors or addition of drugs based on molecular alterations of the tumor cells have been used to achieve pure cancer cell populations. However, these approaches are not universally applicable and vary greatly between cancer types but also between samples within the same cancer type (Sachs et al., 2019;Wallaschek et al., 2019). Such approaches however should be avoided as they will reduce heterogeneity by eliminating subsets of tumor clonesand may stimulate the outgrowth of others which will result in a reduced tumor representation and ability to predict treatment response. Future studies, including single-cell sequencing of organoids should be conducted to investigate how such counter selections affect tumor representation. Second, the derivation time of most cancer organoids is currently still weeks to months. If cancer organoids were to be used as co-clinical avatars this derivation time needs to be shortened to be of actual clinical value to the patient. Third, at the moment most patients die from metastatic disease. Heterogeneity between the primary tumor and developed metastases, is an important cause of treatment failure in the metastasized setting. The opportunity to derive cancer organoids form different tumor sites provides the opportunity to select treatment options to which all distinct tumor location share sensitivity. Multiple studies have successfully used this approach and showed differential as well as similar treatment responses for several anti-cancer agents between organoids derived from multiple tumor sites (Hubert et al., 2016;Tiriac et al., 2018;Li et al., 2019b;de Witte et al., 2020). In clinical practice, taking multiple biopsies from one tumor or taking biopsies from multiple metastases might however not always be feasible which could potentially limit this application into the clinic.

Incorporation of the tumor micro-environment in cancer organoids

Cells from the tumor micro-environment, such as stromal cells, immune cells and endothelial cells are lacking from cancer organoids. This may limit the utility of patient cancer organoids as predictors of treatment response as the tumor micro-environment is a key determinant of therapeutic outcome and a potential therapeutic target (Joyce, 2005;Junttila and de Sauvage, 2013). Importantly, hypoxia, a common characteristic of the tumor micro-environment of solid tumors which greatly contributes to malignant behavior and chemo- and radioresistance does develop in organoids once they reach a certain size (Hubert et al., 2016). The importance of the tumor micro-environment has also been shown by incorporating cancer-associated fibroblasts in pancreatic cancer organoids which led to increased resistance towards treatment (Liu et al., 2020).

Several attempts have already been made towards incorporating aspects of the tumor micro-environment into the cancer organoid system (Bar-Ephraim et al., 2020). These include co-culture of tumor cells and immune cells or the preservation of original tumor micro-environmental components in the culture system (Votanopoulos et al., 2019). An example of this is the development of a co-culture system of peripheral blood mononuclear cells (PBMCs) and NSCLC or CRC cells. In this system, autologous tumor-reactive T-cells could be induced which were also shown to specifically kill tumor organoids whereas matched healthy airway organoids were unaffected (Dijkstra et al., 2018). An air-liquid-interface (ALI) system was used to propagate cancer organoids directly from human or mouse tumor biopsies with preservation of the immune stroma and original tumor T-cell receptor spectrum and used to model immune checkpoint blockade therapy (Neal et al., 2018). In another example, glioblastoma biopsies were cut into small pieces without using a BME. This approach showed retention of immune and endothelial cells during culture and could be used to test for CAR-T cell treatment. However, whether these immune cells remain functional after prolonged culture is still uncertain (Jacob et al., 2020).

Whereas endothelial cells can be preserved in acute slice culture systems and by starting a culture directly from a tumor biopsy without disturbing the tissue architecture, re-creating functional blood vessels requires a very different approach. Towards this goal, human vascularized brain organoids by co-culturing brain organoids and in vitro differentiated iPSCs towards endothelial cells were generated

(Pham et al., 2018). Furthermore, tumor-on-a-chip models have been developed that include a microfluidic model to mimic vasculature and a running blood stream to further mimic the in vivo drug delivery situation (Ayuso et al., 2019).

Drug response standardization in cancer organoid studies

In order to fully understand the potential of cancer organoids as patient avatars important aspects need to be addressed. Most studies to date have used arbitrary drug dosages or titration curves. While this is a fairly common approach in pre-clinical research, it is recommended to use human equivalent dosages (i.e. measured drug concentrations in cancer tissue in vivo) in treatment experiments using cancer organoids. These differences in drug dose could lead to survival of cell populations in vitro that do not die in vivo or vice versa. Furthermore, since all studies use different drugs schedules and dosages, discrepancies of the predictive value of organoids between different studies can occur. Changes in drug concentration due to differences in diffusion of metabolites, in vivo drug metabolism and limited drug penetrance as a result of physiological barriers such as the blood-brain-barrier (BBB) can change the efficacy of a treatment option due to inadequate drug concentration in the cancer organoid. Microfluidics and an artificial BBB using organ on a chip technology may advance the field in this respect (Ayuso et al., 2019;Ahn et al., 2020). Furthermore, anti-cancer drugs are administered in specific treatment schedules in clinical practice. Especially concurrent treatment with multiple systemic agents and/or radiotherapy is still lacking in most cancer organoid studies whereas this is a common treatment strategy in daily clinical practice. Another important consideration that requires critical analysis are the treatment endpoint assays used in cancer organoid studies. It is well established that short term proliferation and viability /cell death (apoptosis, BrdU, ATP assays) are not predictive for long term survival and tumor control probability in response to radiotherapy and chemotherapy. Organoids are composed of heterogeneous cell population in which cancer stem cells have different responses to treatment and constitute only a minor fraction of the organoid population. Therefore, ninety percent cell death may not involve the most resistant clones within the organoid or the tumor in the patient. As tumor stem cells are intrinsically more resistant to treatment (Baumann et al., 2008;Cojoc et al., 2015) than bulk -non tumor stem cells- treatment schedules should also include long term survival assays (e.g organoid replating studies, clonogenic assays) combined with tumor stem cell biomarkers to be more predictive for the tumor cure dose in vivo.

Development of recurrent cancer organoids

An interesting application of cancer organoids to be further explored is the development of organoids from recurrent cancer. Some studies describe organoids from biopsies from recurrent tumors, both with and without matched primary tumors (Lee et al., 2018;Kopper et al., 2019). Another interesting approach could be the in vitro development of a recurrent organoid from primary tumor organoids (Buzzelli et al., 2018). Under selection of treatment pressure, resistant subpopulations within the tumor could potentially outgrow the treatment-sensitive cell populations or cause the acquisition of new mutations that cause resistance or may identify new therapeutic targets. Future studies should address if in vitro induced resistance mechanisms mimic the behavior of recurrent disease in patients.

Corresponding cancer- and healthy tissue organoids

Another important advantage of organoids is the possibility to simultaneously culture cancer and healthy tissue organoids from the same patient. Several studies have already implemented this technique in which it supported selection of the most promising treatment option (Dijkstra et al., 2018;Hou et al., 2018;Ponz-Sarvise et al., 2019;Calandrini et al., 2020). Normal tissue toxicity is one of the main dose-limiting factors in cancer therapy, and being able to predict this can be of great benefit towards both optimal cancer treatment as well as maximizing quality of life. Thus, organoids are not only useful to individualize treatment in order to eradicate cancer cells but also to exclude potentially toxic treatments. The use of normal tissue organoids in parallel is only just emerging but would be a key feature to the armamentarium of organoids as clinical avatars for personalized precision medicine.

Conclusion

In conclusion, cancer organoids exhibit the potential to act as predictors for clinical treatment response. However, quantitative data to make accurate statements about their predictive value is mainly lacking from current studies which is warranted to work towards their clinical implementation.

Biobanks of cancer organoids can be used to identify targetable mutations and patient subgroups to stratify patients for specific anti-cancer treatment options, beyond single predictive molecular markers. This approach is currently utilized by actively involving cancer organoids in ongoing clinical trials to improve patient selection (NCT03416244; NCT03307538).

Finally, cancer organoids can act as living surrogates (patient avatars) to use for high-throughput drug screening approaches to aid directly in the treatment of the specific

patient that the organoid has been derived from and for the discovery of novel drug and targets. This possibility opens up major possibilities in the selection of personalized treatment options and the prevention of normal tissue toxicity.

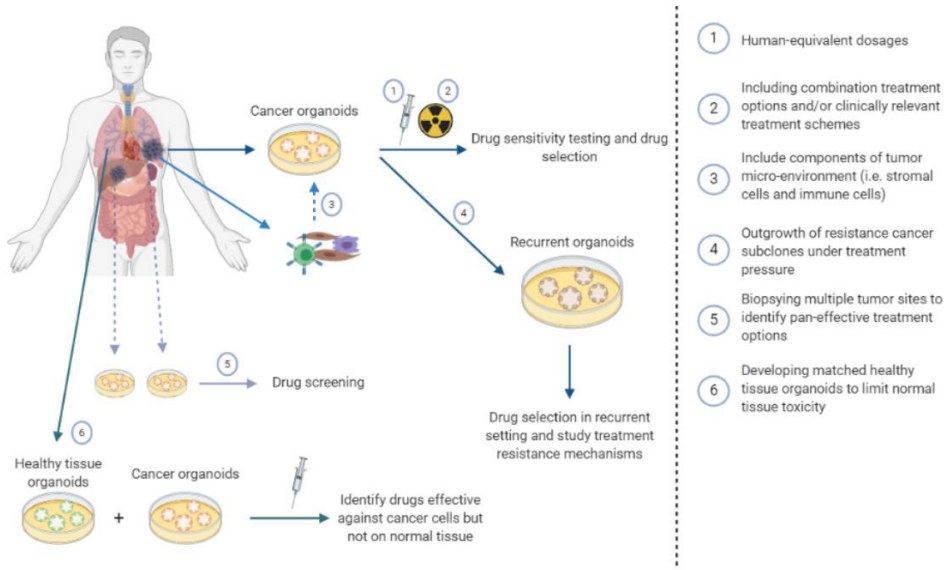

Figure 1. Potential applications of cancer organoids to improve treatment prediction and clinical applicability.

Figure 1 was created using Biorender.com.

Acknowledgments

Contribution to the Field Statement

The culturing of primary cancer organoids from patients has exploded in the past years and for most cancer types, organoid models have been developed. Initial studies have described the technical challenges and characterization of these organoids, but now many studies address the predictive benefit of cancer and normal tissue organoids as patient avatars, the next step in personalized cancer care. This review describes the studies in which cancer organoids have been used to test the response towards standard-of-care treatments as well as novel therapeutic options. Importantly, we review co-clinical studies in which the cancer organoid treatment response can be directly correlated towards the individual treatment response. Although these first studies are promising, only a few studies have been able to derive actual quantitative

predictive values for cancer organoids which are needed to assess the clinical value of cancer organoids in selecting the most appropriate treatment option. We also critically evaluate the current status of cancer organoids and discuss current limitations and potential applications that have to be taken into account to take cancer organoids a step further into the clinical decision making process.

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Chapter 3

Modelling tumor heterogeneity in glioblastoma using patient-derived tumor organoids

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Chapter 4

Non-invasive glioblastoma testing: multimodal approach to monitoring and predicting treatment response

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Abstract

Glioblastoma is the most aggressive adult primary brain tumor which is incurable despite intensive multimodal treatment. Inter- and intratumoral heterogeneity poses one of the biggest barriers in the diagnosis and treatment of glioblastoma, causing differences in treatment response and outcome. Non-invasive prognostic and predictive tests are highly needed to complement the current armamentarium. Non-invasive testing of glioblastoma uses multiple techniques that can capture the heterogeneity of glioblastoma. This set of diagnostic approaches comprises advanced MRI techniques, nuclear imaging, liquid biopsy and new integrated approaches including radiogenomics and radiomics. New treatment options such as agents targeted at driver oncogenes and immunotherapy are currently being developed, but benefit for glioblastoma patients still has to be demonstrated. Understanding and unraveling tumor heterogeneity and micro-environment can help to create a treatment regime that is patient-tailored to these specific tumor characteristics. Improved non-invasive tests are crucial to this success. This review discusses multiple diagnostic approaches and their effect on predicting and monitoring treatment response in glioblastoma.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor with an incidence of 2-3 cases per 100.000 people [1]. Currently a median survival of approximately fourteen months is achieved with intensive multimodal treatment. However, despite this intensive treatment there is no cure and recurrence of GBM is inevitable [2].

Diagnosis and treatment

Diagnostic approaches in GBM are rapidly evolving. The diagnosis is currently based on the recently revised WHO criteria (2016) for the classification of central nervous system tumors. [3]. At present, histopathological investigation of a tissue sample from a suspected GBM lesion is the gold standard for the diagnosis. This is currently complemented by molecular diagnostics of which identification of O6-methylguanine-DNA-methyltransferase (*MGM*T) methylation, isocitrate dehydrogenase (*IDH*)-mutation and *1p19q* co-deletion are currently the most valuable in daily clinical practice. Methylation of the *MGMT* gene is the only predictive marker for treatment response available and is predictive of an improved response to alkylating chemotherapy such as temozolomide [4, 5]. The *IDH*-mutation status [6] and 1p19q co-deletion [7] are of prognostic value but do not predict treatment response in patients with GBM. These markers are however of predictive value in oligodendroglioma [8, 9]. .Additional markers such as telomerase reverse transcriptase promotor (*TERT*) mutations and alpha-thalassemia syndrome X-linked (*ATRX*) can already be used additionally in the classification of GBM subtypes [10, 11].

At the moment, the treatment schedule consists of neurosurgery, concurrent chemoradiation therapy and adjuvant temozolomide. This provides a median progression free-survival of almost 7 months [2]. There is no standard treatment for the recurrent setting. Systemic treatment options include a temozolomide rechallenge, lomustine and anti-angiogenic therapy such as bevacizumab. However effectiveness of these treatment options is limited. Additionally, re-irradiation and re-resection can be considered depending on the localization of the tumor and condition of the patient [12].

Monitoring treatment response

Both during and after treatment for GBM, magnetic resonance imaging (MRI) is the main modality used in the follow-up and monitoring of treatment response. Difficulties arise in monitoring response when it comes to the differentiation between pseudoprogression, radiation necrosis and actual tumor progression. Pseudoprogression is a physiologic post-treatment-related reaction of brain tissue,

based on vascular and cellular injury from chemoradiation therapy. This leads to inflammation and increased permeability of the blood brain barrier (BBB), causing an increase in contrast enhancement on MRI suggestive of tumor progression but without tumor recurrence [13, 14]. Radiation necrosis is a direct effect of radiation therapy, which can mimic tumor progression on imaging techniques but does not reflect actual progression of the tumor. Timing of MRI changes can help to differentiate between pseudoprogression, which occurs most commonly in the first three to six months post-treatment, and radiation necrosis which occurs six months to several years after treatment [15].

Markers are also needed for monitoring treatment response for patients treated with immunotherapy. On MRI, these patients may first show an increase in size or even the formation of new (pseudo)lesions due to the anti-tumor mediated immune response and localized inflammation, which does not necessarily define progressive disease [16, 17]. At the moment, differentiating pseudoprogression from actual tumor progression remains difficult and currently only follow-up imaging with conventional imaging methods is available to define this. Therefore, new imaging techniques and/or biomarkers to further characterize the origin of the imaging changes that are observed are needed to overcome these challenges.

On the other hand, another phenomenon on MRI called pseudoresponse can also occur, mainly during treatment with vascular endothelial growth factor (*VEGF*) signaling pathway modifying agents such as bevacizumab. Bevacizumab induces a steroid-like effect by normalizing the permeability of the BBB, leading to a rapid decrease in contrast enhancement. Thus, while the imaging reflects reduced contrast and suggests a post-treatment response, the effects on overall survival are minimal [13, 18, 19].

Tumor heterogeneity

Tumor heterogeneity poses one of the most important challenges in the current diagnosis and treatment of GBM and it is one of the main difficulties when it comes to finding new treatment options. GBM shows varying tumor characteristics both between patients as well as within individual tumors [20, 21]. Current histological analysis cannot capture the full spectrum of genotypic and phenotypic characteristics, especially when only a single biopsy can be taken.

The intratumoral heterogeneity poses a great challenge in predicting sensitivity and resistance to systemic therapies. As one clone within the tumor may be sensitive to one form of treatment, another clone might harbor certain resistance mechanisms to this treatment in its specific tumor micro-environment. Intratumoral heterogeneity is a dynamic process which changes over time and during treatment which poses challenges in the recurrent setting of GBM, as research has shown recurrent tumors usually show resistance to the traditional treatment options and expresses different mutations when compared to the original tumor [20, 22].

Since conventional MRI cannot reflect tumor heterogeneity, before this can be used as a parameter in daily clinical practice, improved diagnostic approaches should be developed to identify this heterogeneity.

Non-invasive glioblastoma testing

Non-invasive glioblastoma testing (NIGT) combines non-invasive (i.e. non-surgical) techniques to represent the tumor as a whole and provides information on driver mechanisms and tumor micro-environment, all of which are factors that can/should be incorporated into the treatment regime. This can be especially helpful in the selection of patients in order to better predict response to new therapeutic targets, as the current options available are limited. Also the brain is less easily accessible for taking repeated biopsies, which stresses the need for non-invasive approaches. New integrated approaches such as radiogenomics and radiomics can also be an important part of NIGT. Radiogenomics is an experimental diagnostic and predictive tool which studies the association between (qualitative) imaging features and molecular markers [23]. Radiomics on the other hand uses a computational analysis to extract quantifiable information about the underlying tumor characteristics by high-throughput mining of large amounts of quantitative features from images based on for example textures, intensities and shapes [24, 25]. Radiomics has already been more extensively studied in for example head-and-neck cancer [26, 27] and lung cancer [28].

The objective of this review is to discuss the already established approaches as well as future diagnostics used for monitoring and prediction of treatment response in patients with GBM by creating a so-called NIGT platform. This includes a multimodal approach to fully capture the complexity and heterogeneity of GBM with use of conventional techniques such as imaging techniques, enhanced by computational approaches and the use of circulating biomarkers.

Magnetic Resonance Imaging

MRI is clinically used in diagnosis and follow-up of cerebral tumors. The use of imaging to predict patient survival has been applied since as early as 1996 [29, 30]. Features found to be correlating with a longer survival in GBM are the presence of non-enhancing tumor and the absence of either edema, satellites or multifocality [31].The Visually Accessible Rembrandt Images (VASARI) Research Project aimed to make MRI features more accurate and reproducible. In this project, a set of 24 observations describing the morphology of brain tumors on contrast-enhanced MRI were reported and analyzed for their prognostic significance on overall survival [32, 33].

Monitoring treatment response

For the evaluation of tumor response after first-line treatment, the Response Assessment in Neuro-Oncology (RANO) criteria of 2010 is currently used [34]. A major drawback of these criteria is its non-volumetric criteria and lack of use of advanced MR techniques. For instance, with using only these RANO criteria, pseudoprogression cannot be distinguished from radiation necrosis or disease recurrence [35].

Several advanced MR techniques have been developed to improve standard contrast-enhanced MRI, such as diffusion weighted imaging (DWI), perfusion imaging and magnetic resonance spectroscopy (MRS) [15]. DWI displays the cellularity within tissue by detecting free diffusion of water molecules. The apparent diffusion coefficient (ADC) is a derived DWI parameter in which the T2 signal from the original DWI is excluded to overcome the so-called 'T2 shine-through effect', which causes a high signal on DWI that is not due to restricted diffusion. DWI and ADC are widely used in tumor imaging, where a decrease in ADC signal has been shown to correlate with increasing tumor cellularity while an increase in signal correlates with decreasing cellularity as a result of successful treatment [36, 37].

A relatively new DWI technique that has been developed is functional Diffusion Map imaging (fDM), which reflects differences in ADC signal over time. This fDM analysis has shown to be able to distinguish progressive tumors from stable and partially responsive tumors [38, 39]. Although this technique is promising, there is a great variability in protocols collecting and processing DWI/ADC data between different vendors, standing in the way of wide scale use [40].

Perfusion images can be acquired in various ways, with dynamic susceptibility-weighted contrast-enhanced MR (DSC MR) being most widely used. Other perfusion techniques include dynamic-contrast enhanced MR (DCE MR), which is comparable to DSC MR, and arterial spin labeling (ASL) perfusion, which does not require intravenous contrast but is more susceptible for artifacts. DSC MR is able to assess cerebral microvasculature by following an administered contrast agent as it passed through the microvasculature. Tumors tend to have a higher number and larger volumes of blood vessels. Furthermore, remodeling of the extracellular matrix disturbs the BBB and causes leakage of contrast [14, 41, 42]. By comparing e.g. tumor areas with healthy brain tissue, relative cerebral blood volumes (rCBV) can be measured [14, 41]. The presence of high rCBV has been shown to represent active neovascularization and viable tumor, whereas normal rCBV in apparent lesion progression could point to e.g. chemoradiation effects and thereby exclude pseudoprogression and radionecrosis [43, 44].

MRS can be used to measure the distribution of chemical metabolites in brain tissue and thereby identifying differences in metabolic turn-over of brain tissue. As high-grade tumors are highly metabolically active and are accompanied by a leaky blood-brain barrier, regional differences can be found in the spectroscopic profile in tumor depositions, compared to necrosis, pseudoprogression and healthy brain tissue. In ^{1}H-spectroscopy elevated peaks of lipid, lactate, choline, and myoinositol and reduced NAA signal are typical findings in primary brain tumors [45, 46]. Due to patient and tumor specific differences an unequivocal threshold of metabolic signal ratios cannot be determined making it difficult to establish uniform guidelines and accuracy, however MRS changes in time can be of help to strengthen suspicions on for example tumor progression or response [47]. MRS alone therefore has a moderate diagnostic performance in differentiating glioma recurrence from radiation necrosis and should always be combined with other advanced imaging technologies [48].

Tumor heterogeneity and predicting treatment response

GBM is subdivided into four subcategories based on histopathological features and specific mutations and molecular markers: proneural, neural, mesenchymal and classical subtypes. Each subset is associated with specific mutations; therefore identification of the subtype by radiogenomics can provide information on driver mechanisms in the tumor. Between these subtypes, the proneural subtype is thought to have the most favorable prognosis [49]. Also different subtypes react differently to different treatment options [50]. Radiogenomics can be applied to predict the GBM subtype. Volumes of both contrast enhancement and necrosis are higher in tumors with the mesenchymal subtype compared to the proneural subtype. GBMs with less than 5% tumor enhancement are mostly of the proneural subtype. On the other hand GBMs with less than 5% non-enhanced tumor rarely represent proneural tumors and are more linked to the classical or mesenchymal subtype [51, 52].

Radiogenomics can also be used as tool to predict mutational status. *IDH1*-mutational status is associated with a localization of the tumor in the frontal lobe, a higher percentage of non-contrast enhancing part of the tumor and the presence of cysts on MRI [53, 54]. MRS has recently been used to predict *IDH*-mutation status. MRS can measure elevated levels of 2-HG metabolite which is a surrogate marker for *IDH* mutated tumor cells, and can correctly identify *IDH* mutation status in 88.6% of patients (sensitivity 89.5%, specificity 81.3%). However further technical improvement of this technique; including voxel localization, as well as understanding of the impact of tumor heterogeneity on MRS is needed before it can be used in daily clinical practice [55, 56].

MGMT-methylated tumors tend to be lateralized to the left temporal lobe whereas *MGMT*-unmethylated tumors are more frequent in the right hemisphere. This may be due to asymmetry in brain structure, function and gene expression between the hemispheres [57]. *MGMT*-unmethylated tumors have a higher percentage of tumor enhancement and T2/FLAIR hyperintensity when compared to *MGMT*-methylated tumors [53]. Several imaging features are potential indicative of *MGMT*-methylation such as, mixed nodular enhancement, limited edema and moderately increased rCBV [23].

The presence of the *1p19q* co-deletion is linked to classical oligodendroglial MRI characteristics such as heterogeneous T2 signal intensity and the presence of calcifications. Advanced imaging techniques have not yet shown to improve the capacity to identify the *1p19q* co-deletion over conventional MRI to identify oligodendroglial tumors [23].

Epidermal growth factor receptor (*EGFR*) amplification is associated with a significant higher percentage of contrast enhancement and T2/FLAIR hyperintensity compared with tumors lacking *EGFR* amplification. Also, *EGFR*-amplification and *EGFRvIII* mutant GBMs are commonly associated with localization in the left temporal lobe [53].

Apart from already mentioned molecular markers others known driver genes, such as phosphatase and tensin homolog (*PTEN*), platelet-derived growth factor receptor A (*PDGFRA*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), retinoblastoma 1 (*RB1*) and tumor protein 53 (*TP53*) are also under investigation and significant image correlations for these genes have already been identified [58].

The aforementioned advanced imaging techniques can also aid in exploring tumor heterogeneity. Both CBV and ADC measurements are found to be influenced by

tumor aggressiveness and it is suggested that the heterogeneous genetic and cellular expression patterns within GBM influence anatomic and physiologic MR imaging [59]. These techniques can also guide neurosurgeons in determining the biopsy location.

MRI-based radiomics for GBM is a relatively new area for which little research has been published to this date. A study of 82 GBM patients reported favorable results in the performance of texture features in predicting the molecular subtype and 12-month survival [60]. For the prediction of 12-month survival based on pattern analysis sensitivity and specificity of 0.86 and 0.64 are reported. The prediction of GBM subtype was also investigated. Accuracy for classical, mesenchymal, neural and proneural subtypes were 0.88, 0.70, 0.85 and 0.93 respectively [61]. Another study used machine-learning techniques and found an accuracy of almost 80% in predicting overall survival and an accuracy of 76% in predicting the molecular subtype [62].

MRI texture analysis has been found to be able to facilitate in characterizing intratumoral heterogeneity and may therefore aid in identifying genetically different components of the tumor and understanding its consequences for prognosis, treatment sensitivity and resistance [58]. It has been shown that radiomics features are able to visualize spatial gene-expression within a tumor [63]. Patients can be subdivided into different clusters using texture features. For example, one study divided patients into the 'pre-multifocal', 'spherical' or 'rim-enhancing' cluster based on quantitative imaging features. Each of these clusters was linked to different signaling pathways and microarrays and has been shown to be prognostic for survival [64].

Radiomics was found to have prognostic value for both survival and progression in patients with recurrent GBM receiving bevacizumab. Therefore it might be possible to develop pre-treatment biomarkers based on radiomics to predict benefit from bevacizumab [65, 66]. These findings illustrate the possibilities of applying radiomics in the prediction of treatment response in patients with GBM. Further optimization of this technique and validation of radiomics profiles as predictors for different mutation statuses and/or survival is needed before it can be used in clinical practice.

Nuclear imaging

Molecular imaging by the use of positron emission tomography (PET) is increasingly being implemented into clinical practice for treatment planning and response monitoring of GBM. The most common is fluorodeoxyglucose (^{18}F-FDG) PET imaging; however, compared to other organ systems, ^{18}F-FDG-PET imaging of brain tumors presents unique challenges because of the high background glucose metabolism of normal gray matter masking detection of malignant lesions. Thus, the use of ^{18}F-FDG-PET in brain tumors albeit is limited, although in high grade glioma, ^{18}F-FDG-PET imaging can be used to identify metabolically active disease which correlates with tumor grade [67, 68]. Because of the limited utility of ^{18}F-FDG-PET, the RANO working group has recommended the use of radio-labeled amino acid tracers for PET (AA-PET) instead [69].

Amino acid tracers such as O-(2-[^{18}F]-fluoroethyl)-L-tyrosine (^{18}F -FET) and L-[Methyl-^{11}C]methionine (^{11}C-MET) are currently applied in clinical practice in GBM. Both ^{18}F-FET and ^{11}C-MET show rapid uptake into tumors and can be visualized with high contrast [70]. ^{11}C has a short half life time of 20 minutes, making it less useful for clinical practice. ^{18}F has a much longer half life time (120 minutes) and is only taken up by cells through specific L-transporters -LAT2- that are highly and predominantly expressed on glioma tumor cells. This ensures a high and selective uptake of the tracer in tumor tissue, with a low to negligible background signal in normal brain tissue or in surrounding inflammatory areas. Thus, ^{18}F-FET-PET has a high sensitivity and specificity for the detection of malignant gliomas [71-74]. A biopsy-controlled study has shown that with a combination of MRI and ^{18}F-FET-PET a sensitivity of 93% and a specificity of 94% can be achieved [71]. Other amino acid tracers currently under investigation include ^{18}F-FDOPA (phenyl alanine) PET, a dopaminergic tracer, and alpha^{11}C-L-methyl-tryptophan PET, a tryptophan analog.

Monitoring treatment response

^{18}F-FET-PET currently has multiple potential clinical applications including the monitoring of treatment response and can distinguish tumor recurrence from radiation necrosis or pseudoprogression [69]. A study investigating ^{18}F-FET for PET-guided radiotherapy concluded that size and geometrical location of gross tumor volume and biological tumor volume, defined by ^{18}F-FET uptake, were significantly different in patients where the biological tumor volume extended up to 10-20 mm from the margin of contrast enhancement on MRI, potentially improving local tumor control due to improved radiotherapy planning using ^{18}F-FET-PET [75].

Multiple studies have shown that both ^{18}F-FET-PET and ^{18}F-FDOPA-PET have a higher diagnostic accuracy than conventional MRI in differentiating glioma recurrence from post-treatment tissue changes. For example, studies show a sensitivity and specificity of 92.3% and 44.4% respectively for MRI compared to 100% and 88.89% ^{18}F-FDOPA PET [76-79]. A prospective study investigating the predictive value of ^{18}F-FET-PET in patients treated with chemoradiation has shown that a decrease of ^{18}F-FET-PET accumulation reflects tumor response to the therapeutic intervention at an early stage of the disease and predicts outcome, whereas contrast-enhanced MRI did not [78].

Tumor heterogeneity and predicting treatment response

Correlation between different types of AA-PET standard uptake values (SUV) and molecular markers, in the context of radiogenomics, is currently under investigation. A longitudinal prospective study has investigated ^{18}F-FET-PET as an imaging biomarker and they concluded that the biological tumor volume before treatment was a strong prognostic marker for both overall- and progression-free survival independent of treatment as well as *MGMT* promoter methylation, and other patient-and tumor-related factors. Moreover, tumor uptake kinetics before and after treatment (i.e. TAC curves) are correlated with progression-free survival [80].

A recent study demonstrated the relationship between ^{11}C-MET-PET and *IDH1*-mutation, and found that SUVmax and SUVratio were inversely correlated with *IDH1* mutation [81]. Moreover, a study which combined MRI and alpha[C-11]-L-methyl-tryptophan PET imaging showed prognostic imaging factors such as T1-contrast/PET volume ratios and metabolic volume, which are associated with *EGFR* amplification and *MGMT*-methylation status [82]. To assess the potential of radiogenomics as a diagnosis and predictive tool, well defined preclinical models with specific driver mutations are needed that can be used to validate the sensitivity and specificity of radiogenomics. The ability to identify prognostic or molecular response markers based on imaging features derived from routine diagnostic procedures (MRI, PET, computed tomography (CT)) provides an attractive way of predicting treatment response in GBM.

Tumor hypoxia is a common feature of the tumor microenvironment in GBM and contributes to increased malignancy, poor prognosis and resistance to radiotherapy and alkylating chemotherapy such a temozolomide [83-85]. Acute and chronic hypoxic areas fluctuate in human tumors and contribute to spatial and temporal intratumoral heterogeneity [86, 87]. This has a significant impact on resistance to conventional treatment. Therefore GBM patients might benefit from hypoxia targeting drugs [21]. Molecular imaging of tumor hypoxia could aid in the selection

of patients with hypoxic tumors, which could benefit from specific anti-hypoxic therapies. The efficacy of anti-hypoxic treatments will depend on the presence of hypoxia. Several 2-nitroimidazoles, labeled with ^{18}F have already been investigated in patients to identify hypoxia [88]. In extensive pre-clinical models and clinical trials ^{8}F-HX4-PET has shown to be a promising and non-toxic probe for hypoxia [89-91]. Repeated hypoxia-imaging during the course of disease and treatment will demonstrate the extent of spatial and temporal fluctuations in tumor hypoxia and is likely important in scheduling hypoxia-modifying drug in combination with conventional treatments.

Liquid biopsy

Liquid biopsy (LB) has entered clinical practice in the treatment of several cancer types, including breast and colorectal cancer [92, 93]. LB studies circulating biomarkers which refer to measurable biological molecules found in blood, urine and or other body fluids, like cerebrospinal fluid (CSF). Although LB has refined the individual treatment for several cancer subtypes, relatively little progress has been made with regards to validation of circulating biomarkers for primary brain tumors. Nevertheless, although the translation of biomarker development into neuro-oncology is lagging behind compared to general oncology, the prerequisites for adequate extrapolation are present, mostly in experimental studies [94]. In these studies, circulating tumor cells (CTCs), circulating free nuclear acids (cfNA), extracellular vesicles and circulating proteins and metabolites have been described.

For brain tumors, where non-invasive procedures are complex, precarious and may be non-representative for outcome, circulating biomarkers pose a realistic option. For the inoperable patients, which mostly occurs in the recurrent setting, circulating biomarkers could be the source of a molecular profile of the relapsed tumor, allowing clinicians to identify potentially druggable molecular alterations driving recurrence.

Monitoring treatment response

miRNAs are small (about 21-24 nucleotides) non-coding regulatory RNA molecules and can be detected as cell free entities or as the content of circulating extracellular vesicles (EVs) in plasma/serum or CSF. EVs are small nanometer size membrane-enclosed particles that are released from GBM living tumor cells. EVs that can be isolated from both blood and CSF are a rich source of tumor-derived molecules such as DNA, miRNA, mRNA, proteins, lipids and metabolites, because the structure of EVs protects them from nucleases and proteases [95].

The human genome encodes for miRNAs which have been shown to regulate most hallmarks of tumor development and progression via transcriptional silencing or translation inhibition of both oncogene and suppressor genes and have tumor/tissue specific signatures [96]. miRNAs have been described in GBM, mostly from resected specimens. miR-21 is the most reliable plasma biomarker in glioma diagnosis and seems to be valuable distinguishing tumor progression from pseudo-progression or radionecrosis [97]. Exosomal miRNA-21 in CSF of glioma patients has been shown to correlate with glioma recurrence [98]. An increase in levels of blood born annexin V positive microvesicles during chemoradiation is associated with earlier recurrence and shorter overall survival. Since the number of patients included in this analysis was only small (n=16) further investigation is needed [99].

Tumor heterogeneity and predicting treatment response

The ability to detect CTCs in GBM patients has been established [100]. Also, using single cell genome sequencing, some unique mutations were found in the CTCs as well as in the parental tumor [101]. However the most important limiting factor for the clinical implementation of CTCs is their scarcity, which makes it difficult to adequately assess GBM heterogeneity. Also it has not yet been established in GBM if CTCs can be identified which show characteristics of brain tumor initiating cells.

Circulating tumor DNA (ctDNA) is much more abundant than CTCs and contains the mutations present in tumors [102]. In GBM patients a lower rate of ctDNA is detected compared to other solid tumors, mainly due to the only partially disrupted BBB. In several small retrospective studies ctDNAs were successfully detected in GBM patients and multiple molecular alterations were characterized including loss of heterozygosity (LOH) in chromosomes arms *1p*, *19q* and *10q*, *IDH1* and *EGFRvIII* mutations as well as methylation of promoters of *MGMT*, *PTEN* and *CDKN2A* [103-105]. Only few studies have reported on the in plasma half-life of ctDNA. The available data propose that the fast turnover of ctDNA reflects tumor homeostasis [106]. Until now, the clinical utility of candidate ctDNAs as biomarkers for patients with GBM has not been demonstrated and large scale prospective studies are needed before their implementation in clinical practice.

miR-130a was found to positively correlate with temozolomide response in GBM patients, independent from *MGMT* methylation status [107]. miR-603 is another regulator of *MGMT* and could complement assessment of *MGMT* methylation, which alone cannot completely explain temozolomide efficiency, as a predictive marker for treatment response [108]. miR-181d levels in the serum of GBM patients is also

shown to correlate with response to temozolomide; since this miRNA, like miR-603, is directly involved in the downregulation of *MGMT* [109].

Mutant *IDH* enzymes acquire neomorphic enzymatic activity, thereby catalyzing the production of D-2-hydroxyglutarate (D2HG), an oncometabolite that accumulates at high levels and inhibits several enzymes notably involved in histones and DNA demethylation [110]. In most patients with *IDH1*/2 mutant gliomas, plasma D2HG values are in the normal range [111], suggesting limited clinical value of this oncometabolite. However, combining this technique with MRS which can, as mentioned before, measure D2HG metabolite concentrations in brain tissue, might be useful in exploring *IDH* mutation status.

Several circulating proteins have been evaluated and include proteins with cell lineage such as *GFAP* [112], *NCAM* [113] and *S100B* [114], matricellular proteins and matrix metalloproteinases such as *YKL*-40, *MMP2*, *MMP9* [115], *TIMP*-1 and *osteopontin* [116] and cytokines [117], growth factors and growth factor receptors such as *VEGF*, *FGF-2*, *PlGF*, *IGFBP-5*, *EGFR*, *VEGFR1* [118] and *TGF-β1* [119]. Further validation of these biomarkers is warranted.

Discussion and future perspectives

Advanced MR imaging techniques, nuclear imaging, liquid biopsy and integrated radiogenomics and radiomics approaches are examples of non-invasive diagnostic methods to uncover underlying tumor characteristics. GBM is a challenging tumor both from a diagnostic and therapeutic point of view. Recent advances are made when it comes to molecular markers and the understanding of underlying driver oncogenes and tumor micro-environment, all factors which contribute to treatment sensitivity and resistance. Diagnostic methods to accurately identify these factors and their impact on outcome are needed to be able to put this knowledge to clinical use.

Tumor heterogeneity poses a big challenge in the use of targeted treatment approaches. Apart from heterogeneity on the genetic level, non-genetic factors such as the tumor micro-environment also influence the development on cancer cell populations [120]. Different niches have been identified in GBM harboring very different epigenetic and environmental factors which also play a role in treatment resistance and heterogeneity [121]. These differences make it challenging to create one uniform treatment schedule for GBM and a comprehensive insight into the behavior of these distinct tumor cell populations is needed.

The diagnostic modalities previously discussed all have future possibilities to improve the understanding of tumor heterogeneity and the prediction of treatment response as well as the monitoring of treatment response with regards to the differentiation of pseudoprogression, radiation necrosis and actual tumor progression. The latter is especially important when it comes to patients treated with immunotherapy, for which no adequate distinction between pseudoprogression and actual progression can currently be made.

MRI is already well established within the clinic of GBM, further optimization through higher resolutions (e.g. ultra-high field MRI), wider use of advanced imaging techniques and further research, including clinical validation, on the application of radiogenomics will improve the diagnostic power of MRI. The same applies to nuclear imaging, especially different types of AA-PET for which additional studies on known amino acid tracers and the development of new tracers to improve diagnostic accuracy, both in the setting of the primary diagnosis as well as in the monitoring of treatment response and patient follow-up, is warranted. Radiomics is an important topic in different types of solid tumors and still relatively new in GBM. Radiomics analysis is one of the most promising techniques for the differentiation between different GBM subsets and in evaluating and monitoring intratumoral heterogeneity [122]. Ideally predictive radiomics models are created as predictors for GBM subtypes as has already been established using radiomics in other types of solid tumors [24]. Before radiomics can be applied prospective validation is needed as well as standardization of imaging protocols, imaging segmentation and feature extraction to ensure interoperability of multi-center radiomics studies [123].

Liquid biopsy provides a different approach for understanding tumor characteristics. Further research should focus on determining the clinical value of liquid biopsy and also which liquid source and which biomarker technique to use. Also, the possibility to use liquid biopsy in patients after a tumor resection as a marker for tumor recurrence has yet to be studied. Tumor heterogeneity will remain an important pitfall in liquid biopsy technique; this might be overcome by combining liquid biopsy with other non-invasive markers but will remain a challenge.

Future research should focus on determining the sensitivity and specificity and validating the techniques previously discussed for GBM. Being able to understand and unravel intratumoral heterogeneity provides clinicians with important information to create the most optimal treatment regime and this should therefore be the focus of future studies.

NIGT offers a non-invasive panel to understand the driver mechanisms as potential treatment targets as well as identifying the tumor micro-environment. Combining different diagnostic modalities aims to achieve optimal diagnostic power for identification of tumor characteristics. Understanding the tumor micro-environment (e.g. hypoxia, angiogenesis and immune infiltration) can help in finding new ways to treat GBM or to alter the tumor micro-environment to improve the effectiveness of systemic therapies and radiotherapy. Unique to the brain micro-environment is the BBB which limits effectiveness of therapeutic agents. Advances in imaging allow the visualization of changes in the tumor micro-environment and tissue architecture as a response to treatment and can therefore serve as a marker for treatment response.

Given the limitations of each of the currently available non-invasive tools these diagnostic methods are ideally combined into a so-called NIGT platform: a multimodal non-invasive approach to visualize the tumor and its underlying tumor characteristics in a spatially and temporally relevant manner. Using this NIGT platform, predictive models for GBM can be created, both in the primary and the recurrent setting. This will guide clinicians in selecting the appropriate treatment option treatment monitoring and adaptation in the era of patient-tailored precision medicine.

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Chapter 5

Prognostic and predictive value of integrated qualitative and quantitative magnetic resonance imaging analysis in glioblastoma

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Simple Summary: Glioblastoma (GBM) is the most malignant primary brain tumor for which improving patient outcome is limited by a substantial amount of tumor heterogeneity. Magnetic resonance imaging (MRI) in combination with machine learning offers the possibility to collect qualitative and quantitative imaging features which can be used to predict patient prognosis and relevant tumor markers which can aid in selecting the right treatment. This study showed that combining these MRI features with clinical features has the highest prognostic value for GBM patients, this model performed similarly in an independent GBM cohort showing its reproducibility. The prediction of tumor markers showed promising results in the training set but not could be validated in the independent dataset. This study shows the potential of using MRI to predict prognosis and tumor markers but further optimization and prospective studies are warranted.

Abstract: Glioblastoma (GBM) is the most malignant primary brain tumor for which no curative treatment options exist. Non-invasive qualitative (VASARI) and quantitative (radiomics) imaging features to predict prognosis and clinically relevant markers for GBM patients are needed to guide clinicians. A retrospective analysis of GBM patients in two neuro-oncology centers was conducted. The multimodal Cox-regression model to predict overall survival (OS) was developed using clinical features with VASARI and radiomics features in IDH-wild type GBM. Predictive models for IDH-mutation, MGMT-methylation and EGFR amplification using imaging features were developed using machine learning. The performance of the prognostic model improved upon addition of clinical, VASARI and radiomics features for which the combined model performed best. This could be reproduced after external validation (C-index 0.711 95%CI 0.64-0.78) and used to stratify Kaplan Meijer curves in two survival groups (p-value <0.001). The predictive models performed significantly in the external validation for EGFR amplification (AUC 0.707, 95%CI 0.582-8.25) and MGMT-methylation (AUC 0.667, 95%CI 0.522-0.82) but not for IDH-mutation (AUC 0.695, 95%CI 0.436-0.927). The integrated clinical and imaging prognostic model was shown to be robust and of potential clinical relevance. The prediction of molecular markers showed promising results in the training set but could not be validated after external validation in a clinically relevant manner. Overall, these results show the potential of combining clinical features with imaging features for prognostic and predictive models in GBM but further optimization and larger prospective studies are warranted.

Keywords: glioblastoma; radiomics; MRI; prognosis; prediction; machine learning; survival

Introduction

Glioblastoma (GBM) is the most malignant type of primary brain cancer with an incidence of 2-3 cases per 100.000 [1]. Currently, a median survival of fifteen months is achieved with multimodal treatment [2] with a 5-year overall relative survival of only 6.8% [3]. However, despite this intensive treatment by neurosurgical intervention, concurrent chemoradiation and adjuvant temozolomide (TMZ) [2], GBM is still considered incurable and recurrence is inevitable. Although major improvements in the treatment of cancer have been made, the current standard-of-care for GBM has largely remained unchanged over the past decade.

GBM is diagnosed using gadolinium contrast-enhanced magnetic resonance imaging (MRI) followed by histopathological examination of tumor tissue specimen obtained after either biopsy or resection. Further characterization of GBM has led to the introduction of the 2016 updated WHO classification of central nervous system tumors [4]. This classification integrates histopathological and morphological examination of the tumor with molecular markers [5]. Thus far, the only predictive marker that has been established into clinical practice is the 06-methylguanine-DNA-methyltransferase (MGMT) methylation status, which is predictive of an improved response to alkylating chemotherapy such as TMZ [6]. However, a substantial 'grey-zone' between MGMT methylated and unmethylated patients still exists for which the efficacy of TMZ is still to be determined [7]. Additionally, the presence of a mutation in the isocitrate dehydrogenase (IDH) genes – which has been identified as a positive prognostic marker - is linked to dedifferentiated low-grade gliomas which have a distinctly different clinical behavior compared to IDH wild-type (WT) GBM [8]. Epidermal growth factor receptor (EGFR) amplification is one of the most common genetic alterations (±50%) in GBM [9]. This oncogenic molecular alteration poses a potential therapeutic target but also identifies a biological different subtype of GBM which responds differently to established treatments [10,11]. However, the role of EGFR amplification as a prognostic factor still remains controversial [11-13] and studies using targeted agents for EGFR have so far been unsuccessful but are still ongoing [3]. Additionally, multiple other molecular targets (genetic mutations, amplifications and protein fusion products) have been identified which have either failed in previous clinical trials to improve patient survival or are currently still under investigation [3]. All in all, the integration of molecular markers has led to an improvement in prediction of prognosis and treatment response but a substantial variety remains and no improvement in treatment outcome has been made, which is thought to be due to extensive inter- and intratumor heterogeneity [14].

Intratumor heterogeneity complicates treatment efficacy as different regions within the same tumor may contain cells having distinct genetic compositions, transcriptional subtypes and/or proliferation kinetics [3]. Furthermore, temporal heterogeneity has been observed in which changes in the expression of molecular targets occur over time which limits efficacy of targeted approaches [15,16]. In clinical practice and currently used diagnostic techniques and available prognostic models intratumor heterogeneity is not accounted for; since single-cell sequencing is not routinely used. Additionally, it is not clear if molecular GBM heterogeneity can be captured by qualitative and/or quantitative analysis of imaging features.

Imaging techniques have the advantage over standard pathological examination to also analyze the invasive, non-resected, components of GBM and thus capture and analyze the tumor as a whole. Especially temporal heterogeneity of expression of molecular targets cannot be evaluated using routine clinical diagnostics as re-resection of tumors is not always feasible making non-invasive imaging an interesting alternative. In order to make a standardized analysis of qualitative MR imaging features the Visually Accessible Rembrandt Images (VASARI) features were previously developed [17]. VASARI features include tumor size, location and morphology and have previously shown to be reproducible and of prognostic value [17]. Quantitative imaging analysis using radiomics is an approach to extract imaging features by high-throughput data mining on textures, shapes and intensities [18]. Radiomics has shown prognostic and predictive potential in multiple solid tumors [19,20] including GBM [21]. Furthermore, radiomics features have the potential to analyze the entire tumor and to identify intratumor molecular heterogeneity and underlying biological processes [22,23]. In glioma, radiomics models have been developed to predict tumor grade [24], overall survival (OS) [25] and in GBM trying to predict molecular subtypes [26]. Although IDH-mutation status is established as the best prognostic marker in GBM [27], defining different IDH wild-type GBM prognostic subgroups is still warranted due to their heterogeneous prognosis and clinical behavior.

The main challenge in developing prognostic and predictive imaging-based models is their generalizability towards all GBM patients treated at different centers. Differences in diagnostic techniques (i.e. scanner vendors and protocols) and treatment and population variety can greatly influence model performances [28]. Due to these challenges, this study utilizes two multi-center datasets to train and validate the developed models.

The objective of this study was to investigate the additive value of qualitative and quantitative imaging heterogeneity analysis to established prognostic clinical features. These data were used to develop a prognostic model for OS in a real-world multi-center GBM population for IDH1/2 wild-type (IDH-WT) GBM. Furthermore, the value of imaging features as predictor for clinically relevant molecular markers for GBM was explored.

Results

Patient and tumor characteristics

In total 142 patients were included in the training cohort and 46 patients in the validation cohort. Median OS was 12.0 months (range 0-142 months) in the training cohort and 7.3 months (range 0-30 months) in the validation cohort (log rank p-value 0.001). Patients in the validation cohort more frequently received no adjuvant treatment, but these data were not available for all patients. Patient demographics, received treatment schedules and tumor characteristics are listed in Table 1. Molecular data for a subset of patients is reported as missing due to insufficient FFPE material or poor quality or quantity of extracted DNA. VASARI features were available for all patients in both cohorts. For radiomics analysis T1+Gadolinium and T2- weighted images were available for 105 patients in the training cohort and 44 patients in the validation cohort. MRI characteristics such as types and manufacturers of scanners and imaging protocols are reported in Supplementary Figure 1 and 2. The numbers of patients that were eligible in the two cohorts for the different models are reported in Supplementary Table 1.

Prognostic value of integrative MRI imaging analysis in IDH-wild type GBM population

Median OS was 11.2 months (1.2-132.80 months) in the training cohort and 7.0 months (0.4-29.4 months) in the validation cohort in the IDH-WT GBM population. Univariate Cox-regression analysis of VASARI features for OS in the training cohort resulted in 13 features selected for inclusion in multivariable analysis (Supplementary Table 2). The multivariable Cox-regression model consisted of five VASARI features (Model 1). For radiomics, five radiomics features were selected to predict OS (Model 2)(Table 2). In this study none of the radiomics features showed evidence of a significant correlation with tumor volume (Supplementary Figure 3). Also no significant correlation were found between VASARI, radiomics and clinical features (Supplementary Figure 4). An elaborate explanation of these radiomics features can be found on the Pyradiomics website [29] and in a previous study [30].

Table 1. Overview of patient, treatment and tumor characteristics in the training and validation cohort.

Demographics	Training cohort (N=142)	Validation cohort (N=46)	P-value
Median age at diagnosis (range)	61.4 years (15-85)	61.7 years (18-81)	0.991
Sex (%)	Male: 85 (59.9%) Female: 57 (40.1%)	Male: 29 (63.0%) Female: 17 (37.0%)	0.258
Treatment characteristics			
Surgical treatment (%)	Biopsy: 54 (38.0%) Debulking: 88 (62.0%)	Biopsy: 17 (37.0%) Debulking: 29 (63.0%)	0.112
Adjuvant treatment (%)	STUPP completed: 67 (47.2%) STUPP not completed or Non-STUPP regimen: 75 (52.8%)	STUPP completed: 17 (37.0%) STUPP not completed or Non-STUPP regimen: 16 (34.8%) *Missing: 13 (28.2%)*	0.288
Tumor characteristics			
Isocitrate dehydrogenase (IDH1) (R132H) mutation status (%)	*IDH1/2*-WT: 129 (91.5%) *IDH1*-mutation: 12 (8.5%) *Missing: 1*	*IDH1/2*-WT: 39 (84.8%) *IDH1*-mutation: 5 (10.9%) *Missing: 2*	1.000
Methylguanine methyltransferase (MGMT)-methylation status (%)	*MGMT*-methylated: 37 (26.2%) *MGMT* non-methylated: 104 (73.8%) *Missing: 1*	*MGMT*-methylated: 18 (39.1%) *MGMT* non-methylated: 26 (56.5%) *Missing: 2*	0.045
Epidermal growth factor receptor (EGFR) amplification status (%)	*EGFR* amplified: 47 (37.3%) *EGFR* non-amplified: 79 (62.7%) *Missing: 16*	*EGFR* amplified: 20 (43.5%) *EGFR* non-amplified: 26 (56.5%) *Missing: 0*	0.738

Clinical features that were selected in the clinical model were chosen based on previous studies [31] and clinical expertise (Model 3). Next, VASARI features, radiomics features and clinical features multivariable Cox-regression models were combined in different combinations. Model 4 was developed by combining VASARI PI and Radiomics PI, Model 5 by combining VASARI PI and Clinical PI and Model 6 by combining Radiomics PI and Clinical PI (Model 4-6). Finally, clinical features were combined with the integrated VASARI and radiomics prognostic score to develop an integrated clinical and imaging prognostic model (Model 7)(Table 2). The calibration slope of the PI of Model 7 on the validation set was 0.79 (log-rank test p-value 0.27), indicating there is no certainty for the slope in the validation set being different from 1. The joint test of all predictors with the offsetting of the predicted PI results in the p-value of 0.23, indicating that there is no evidence of a lack of fit on the validation.

Table 2. Multivariate Cox-regression model using VASARI, radiomics and/or clinical features for OS prediction in IDH-WT GBM patients in different prognostic models based on the training cohort (N = numbers of patients used for model development).

Prognostic model variables	Hazard Ratio (95% CI)	P-value
Model 1: VASARI features model (N=129)		
Involvement of eloquent cortex	1.28 (0.88-1.87)	0.198
Multifocality	1.72 (0.97-3.05)	0.064
Subependymal extension	1.75 (1.21-2.53)	0.003
Low proportion of edema	*Reference*	*Reference*
Medium proportion of edema	1.09 (0.75-1.61)	0.653
High proportion of edema	0.45 (0.24-0.83)	0.011
Increased T1FLAIR-ratio	0.59 (0.37-0.94)	0.026
Model 2: Radiomics features model (N=95)		
T1_wavelet.HHH_firstorder_Median	1.04 (0.81-1.3)	0.754
T2_log.sigma.2.0.mm.3D_glszm_LargeAreaLowGrayLevelEmphasis	1.00 (0.83-1.2)	0.958
T2_log.sigma.3.0.mm.3D_glszm_LargeAreaLowGrayLevelEmphasis	1.33 (1.07-1.6)	0.009
T2_wavelet.LLH_firstorder_Mean	1.70 (1.32-2.2)	0.001
T2_wavelet.HHL_glszm_LargeAreaLowGrayLevelEmphasis	0.92 (0.75-1.1)	0.404
Model 3: Clinical features model (N=95)		
Sex (male vs. female)	1.12 (0.70-1.77)	0.644
Type of surgery (resection vs. biopsy)	0.48 (0.31-0.76))	0.002
Age at diagnosis (>70 vs. <70)	1.10 (0.60_2.02)	0.749
Adjuvant treatment (non-STUPP vs. STUPP)	4.92 (2.79-8.67)	0.001
Methylguanine methyltransferase (MGMT)-methylation	0.61 (0.35-1.06)	0.082
Model 4: Integrated imaging model (VASARI + radiomics) (N=95)		
VASARI prognostic score	2.2 (1.4-3.4)	<0.001
Radiomics prognostic score	2.92 (1.9-4.5)	<0.001
Model 5: Integrated VASARI and clinical model (N=95)		
VASARI prognostic score	2.0 (1.3-3.2)	0.003
Clinical prognostic score	2.7 (1.8-3.9)	<0.001
Model 6: Integrated Radiomics and clinical model (N=95)		
Radiomics prognostic score	2.6 (1.7-4.0)	<0.001
Clinical prognostic score	2.8 (1.9-4.1)	<0.001
Model 7: Integrated imaging and clinical model (N=95)		
VASARI prognostic score	2.1 (1.4-3.3)	<0.001
Radiomics prognostic score	3.0 (1.9-4.7)	<0.001
Clinical prognostic score	2.1 (1.4-3.3)	<0.001

To assess the reproducibility performance of the prognostic models, all models were tested on the external validation set (N=38) and the discriminative prognostic value in both cohorts was analyzed using Harrell's C-index (Figure 1A). Model 1 achieved a C-index of 0.61 (95%CI 0.55-0.68) when tested on the whole training cohort (N=129). In order to make a comparison between the different models, the C-index for the VASARI-only model was also calculated using only the patients available in all other models (N=95). In order to visualize the prognostic potential of the integrated imaging and clinical model (Model 7), the data-set was split in a low- and high risk group at a set cut-off value (75th percentile) of the prognostic index in the training cohort. This same cut-off value was applied to the external validation cohort. Two survival groups could be identified (p-value <0.0001) in both the training and validation cohort (Figure 1B-C).

Predictive value of integrative imaging analysis

In order to develop the predictive models for molecular markers (EGFR amplification, MGMT-methylation and IDH1 mutation), the MUMC+ cohort was split into a training (70%) and test (30%) cohort. For the prediction of EGFR amplification, in total eleven VASARI features and four radiomics features were selected in the predictive models using XGBoost machine learning algorithm (Table 3). Both VASARI and radiomics models alone were able to significantly predict EGFR amplification in the test dataset (Figure 2A). In the external validation set both VASARI and radiomics features reached similar results as each other, however an increased predictive value was observed when both models were combined (AUC 0.707 (95%CI 0.582-0.825); Figure 2B-C).

The predictive models developed for MGMT-methylation status consisted of seven VASARI features (logistic regression analysis) and three radiomics features (XGBoost algorithm) (Table 3). VASARI features alone reached similar predictive value in the test and validation dataset with an AUC of 0.668 (95%CI 0.513-0.850) and 0.622 (95%CI 0.475-0.761) respectively. Radiomics features alone could not predict MGMT-methylation in both datasets. An increased predictive value was observed when VASARI features and radiomics features were combined in one predictive model, with an AUC of 0.843 (95%CI 0.696-0.948) in the test dataset but did not perform as wellin the external validation dataset (AUC 0.667 (95%CI 0.522-0.820); Figure 2B-C).

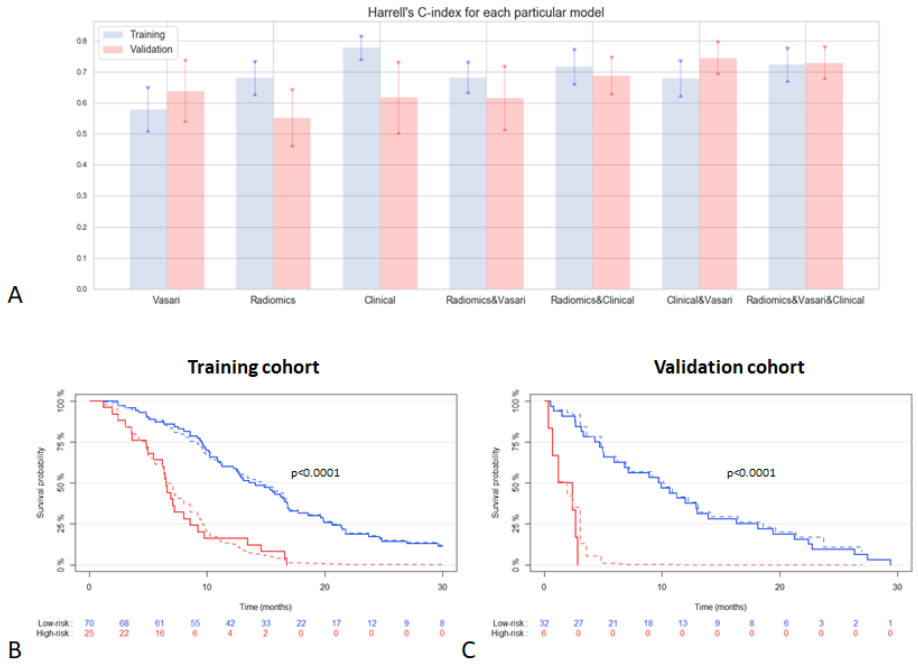

Figure 1. Performance of prognostic models. **(a)** visualization of C-index for all prognostic models (including 95%CI) in the training (N=95) and validation cohort (N=38); **(b)** Kaplan-Meier curve of integrated radiomics, VASARI and clinical model (Model 7) in the training cohort and; **(c)** validation cohort. Low- and high risk groups (blue and red line respectively) cut-off values were determined by set cut-off (75th percentile) in the training cohort. The solid lines represent the observed survival curves, the dashed the corresponding predicted survival curves.

For the prediction of the IDH1 mutation ten VASARI features were included in the multivariate VASARI model and nine radiomics features in the radiomics prediction model developed using the XGBoost machine learning algorithm (Table 3). In the test dataset only radiomics features reached statistical significance with an ROC AUC of 0.816 (95%CI 0.650-0.950), which improved upon combining with VASARI features (Figure 2A). In the external validation set, neither VASARI nor radiomics features or the combination were able to predict the IDH1 status (Figure 2B-C). ROC curves for all predictive models in the training and validation cohort are reported in Supplementary Figure 5 and 6 respectively.

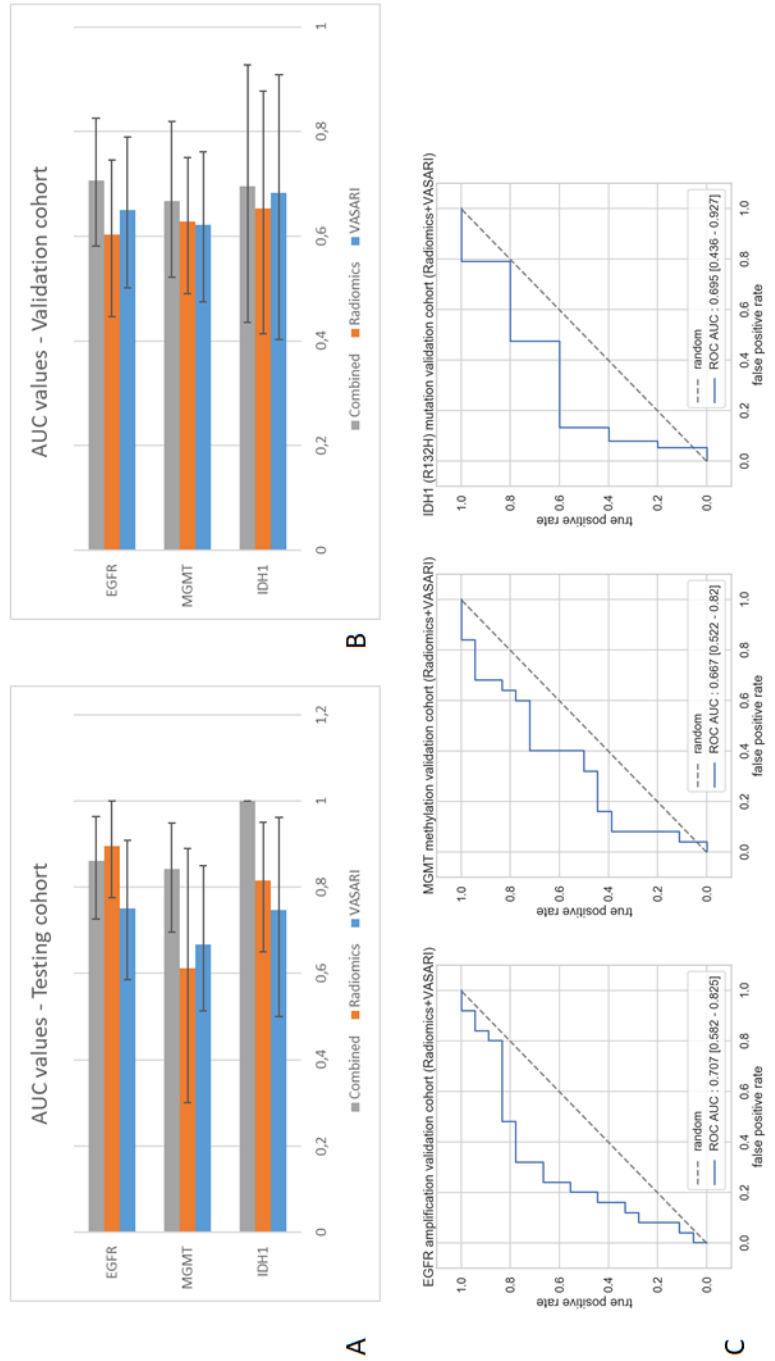

Figure 2. Performance of predictive models; **(a)** Area-under-the-curve (AUC) values and corresponding 95% confidence intervals of different predictive models in the testing cohort and; **(b)** in the validation cohort; **(c)** Receiver operating characteristic (ROC)-curves of combined VASARI and radiomics model predictive performance in external validation set.

Table 3. Selected VASARI and radiomics features in predictive models for *EGFR* amplification, *MGMT*-methylation and *IDH1* mutation in GBM patients in the training cohort.

VASARI features	Radiomics features
EGFR amplification (N=64)	
Size: Major Axis, Minor Axis, Mean Minor Axis, Median Major Axis *Location*: Eloquent location, Midline cross of enhancing tumor *Morphology*: Proportion necrosis *Tumor characteristics*: Hemorrhage, Subependymal Extension, Pial invasion, Definition enhancing margin	*T1+Gado*: wavelet-HLH_glcm_Correlation *T2*: log-sigma-2-0-mm-3D_gldm_LargeDependenceLowGrayLevelEmphasis; wavelet-LLH_glcm_ClusterShade; wavelet-LLH_firstorder_Skewness
MGMT-methylation (N=74)	
Size: Major Axis, Minor Axis, Median Major Axis, Mean Major Axis *Morphology*: Proportion non-enhancing tumor *Tumor characteristics*: Deep white matter invasion, Subependymal extension	*T1+Gado*: no features selected *T2:* wavelet-HLL_gldm_LargeDependenceHighGrayLevelEmphasis; log-sigma-5-0-mm-3D_glrlm_HighGrayLevelRunEmphasis; log-sigma-5-0-mm-3D_glszm_SmallAreaHighGrayLevelEmphasis
IDH1 mutation (N=72)	
Size: Minor Axis, Major Axis *Location*: Tumor side, Eloquent location *Morphology*: Proportion non-enhancing tumor, Proportion Edema *Tumor characteristics*: Pial invasion, Thickness Enhancing Margin, Definition enhancing margin, T1-FLAIR-ratio	*T1+Gado*: wavelet-HLL_glcm_Contrast; wavelet-HLL_glcm_DifferenceAverage *T2*: log-sigma-2-0-mm- 3D_firstorder_90Percentile ; Original_glrlm_LongRunHighGrayLevelEmphasis; log-sigma-3-0-mm- 3D_firstorder_90Percentile; original_firstorder_10Percentile; log-sigma-4-0-mm-3D_firstorder_Uniformity; wavelet-HLL_gldm_DependenceEntropy; wavelet-HLL_glcm_Correlation

Next, histogram heterogeneity was assessed to identify whether radiomics features demonstrate significant differences between the outcome groups in a univariate manner. Only for IDH1 mutation was a significant difference found for two features that could explain the heterogeneity in the outcome. The histograms of heterogeneity for each predictive model and significance values for IDH1-mutation are reported in Supplementary Figure 7 and 8 respectively.

TRIPOD statement and radiomics quality score

The TRIPOD statement adherences was calculated at 77% for this study. The RQS score calculated for this study was 47%. An overview of point allocation towards the TRIPOD statement and RQS score can be found in Supplementary Table 3 and 4 respectively.

Discussion

Increasing curation rates by optimizing treatment strategies is being hampered by the highly invasive nature and GBM specific inter-and intratumoral molecular heterogeneity. MR imaging is currently the preferred diagnostic imaging technique for GBM. However, integrated standardized qualitative and quantitative analysis of different MR sequences has not yet been introduced into prognostic and predictive GBM models. This study retrospectively analyzed two multi-center GBM patient cohorts to develop integrated clinical and imaging prognostic models and predictive models for clinically relevant molecular markers.

Combining clinical features with quantitative and qualitative imaging features resulted in the most optimal prognostic model which could be reproduced in the external validation cohort (C-index 0.72 in training cohort and 0.73 in validation cohort). Despite promising results for predicting EGFR amplification and IDH1-mutation in the test cohort, none of the predictive models for molecular markers were able to predict these markers in a clinically relevant manner in the external validation set.

The prognostic model described in this study is developed for IDH-WT GBM patients as this patient group makes up for the majority of GBM and exhibits large variation in prognosis and treatment response. This variance is also reflected in statistically significant differences in baseline characteristics for OS and MGMT-methylation. However, these differences are also known to exist between centers, in which different treatment decisions and strategies are being implemented. The aim of this study was to investigate the performance of prognostic models in such heterogeneous GBM cohorts. To predict OS, five VASARI features were identified to be of most prognostic relevance. Three of these features are well known prognostic factors and were also previously identified to be negatively associated with OS (involvement of eloquent cortex, multifocality and subependymal extension) and can be attributed to a more invasive growth of the tumor [32,33]. The other selected features, proportion of edema and T1-FLAIR-ratio showed opposite prognostic value in this study when compared to previous studies [33-37]. However, other studies reported no prognostic value for these features and therefore this still remains controversial [32,38].

Radiomics features that were identified to have prognostic value were mainly derived from T2-weighted imaging. This is in line with the hypothesis that the T2-weighted signal corresponds with intratumor heterogeneity and infiltrative tumor growth [39] and this area is accountable for the majority of local recurrences [40]. Therefore,

radiomics features from this area are expected to be of importance for survival prediction as was also shown in previous studies [41,42]. The radiomics signature for OS consists of five features, from which two features are the first order Mean (T2-weighted) and Median (T1-weighted) describing the mean and median intensity values after the LLH and HHH wavelet decomposition of the original MR images. The remaining three features quantify gray level zones in an T2-weighted image, more precisely measuring the proportion in the image of the joint distribution of larger size zones with lower gray-level values after image transformation (Laplacian of Gaussian) which is useful for edge detection. These gray level zone features can potentially be associated with the measure of intratumor heterogeneity [43].

In this study VASARI features alone or radiomics features alone were not able to predict OS in the external validation dataset in a clinically relevant manner. Interestingly, the performance of the prognostic model improved upon combining VASARI, radiomics and clinical features (C-index 0.723 in training cohort and 0.730 in validation cohort) and became clinically relevant. The robustness of this combined model also improved as the model performed similarly in the training- and validation cohort and the uncertainty decreased as represented by a smaller confidence interval of the C-index. Model 5 and 6 report similar performances when compared to the model combining all features. However, the final combined model seems to remain mostly stable between both cohorts, though the actual additive value should be further validated in larger patient cohorts. The combined model was also able to accurately split the two cohorts in a high- and low risk group (p-value <0.001) (Figure 1B-C). Previous studies also observed that combining clinical features with imaging features improves the prognostic value of the model [42,44-47]. The model developed in this study performed similar or better compared to previous findings, even after external validation in a heterogeneous patient cohort. This highlights the clinical relevant potential of combining these features into a multimodal prognostic model which can potentially be applied in clinical practice.

As a proof-of-concept study, this study investigated the capability of VASARI and radiomics features to link phenotype to genotype and predict clinically relevant molecular markers, IDH1-mutation, MGMT-methylation and EGFR amplification, by machine learning approaches. Overall, the predictive models had promising performance on the test set, especially when VASARI and radiomics features were combined (Figure 2A). Unfortunately, none of the developed models were able to predict in the external validation set in a clinically relevant manner with a wide spread in confidence intervals of the AUC values (Figure 2B-C). In order for a model predicting molecular markers to be clinically relevant, much higher AUC values are desired. Since

the presence of the molecular markers has biological consequences on tumor growth and development, specific imaging techniques that reflect biological processes have shown more promising results in the prediction of these markers and should therefore be used for further research. Perfusion-weighted and/or diffusion-weighted MRI features have been used to predict EGFR amplification [48-50] and MGMT-methylation [51], whereas MR spectroscopy [52] and amino acid tracer PET imaging (FET-PET) [53] can predict IDH1 mutation status due to its effects on tumor metabolism.

In addition, by analyzing the heterogeneity histogram for EGFR amplification based on the validation cohort, we can notice that none of the radiomics features has demonstrated significant difference between the outcome groups in the univariate manner. Heterogeneity histogram for MGMT-methylation also did not demonstrate the significant difference between the outcome groups. For IDH1 mutation, however, we can point out a significant difference ($p < 0.05$) for T2_original_firstorder_10Percentile, T1_wavelet_HLL_glcm_DifferenceAverage features, which indicates the ability of these features to reflect the heterogeneity in the outcome (Supplementary Figure 8). These findings also highlight the value of multivariate predictive analysis.

The overall RQS of 47% achieved in this study is higher than generally reported in neuro-oncology radiomics studies [54].

The main strength of this study includes the usage of two independent multicenter datasets. Though the performance of previous prognostic models based on VASARI or radiomics features is generally better, most of these studies only use internal validation methods and lack validation in an independent external dataset [34,55]. The same applies to the performance of predictive models for molecular markers. However, the fact that the promising results for the predictive models in this study in the testing cohort could not be replicated in the external validation cohort stresses the importance of external validation. Additionally, most studies use a more homogeneous patient cohort, for example with regards to treatment characteristics, whereas this present study comprises of two heterogeneous cohorts which more reflects daily clinical practice. For example, corticosteroid usage is known to decrease the amount of edema and therefore altering the T2-weighted signal which can influence both VASARI and radiomics features. Previous studies either do not mention corticosteroid usage or exclude patients using corticosteroids [36,37] even though a significant amount of GBM patients are known to use corticosteroids. Furthermore, multiple studies only use single-institute data in which real-life heterogeneity between MRI acquisition is not represented [56] which is important for the generalizability of radiomics models.

Several limitations should be taken into account when considering the results of this study. The main limitation of this study is the number of patients that were included. Though for the OS models the number of patients is in accordance with the majority of previous studies, especially the limited available molecular data in the external validation set limits the validation capacity of the predictive models. Especially IDH1/2 mutations rarely occur in both cohorts, which is to be expected in GBM, leading to wide confidence intervals and complications in the validation of the model. Future studies using a larger IDH-mutated cohort are needed to accurately test the models developed in this study. Next, the fact that this study is a retrospective study poses a potential selection bias. Additionally, the Karnofsky Performance Score (KPS) is an established prognostic feature which could not be included in this study due to lack of reporting of the KPS in patient files during the time period used for this study. Furthermore, it could be stated that a limitation of this study was the lack of advanced MRI sequences such as diffusion- and perfusion-weighted imaging and PET-MRI. However, this study specifically chose to focus on the relevance of conventional MRI images as these are widely available in clinical centers. Furthermore, MRI radiomics features are known to be dependent on differences in MRI scanners and scanning protocols. The images used in this study were collected from more than ten different hospitals over a ten-year time-period resulting in large differences in technical MRI characteristics. Again, even though this limits the performance of radiomics an ideal prognostic and predictive model should not be dependent on homogeneous data. These differences in MRI acquisition methods are present in the real-life multicenter setting and should be accounted for in order to provide a relevant, clinical applicable model.

In order to further improve the prognostic and predictive potential of non-invasive imaging models several steps need to be taken. First of all, larger – big data - datasets and preferably prospective studies are warranted to develop more accurate and generalizable models. This could pose a challenge, especially in less common types of cancer such as GBM. Next, the first studies on radiomics have been conducted on computed tomography (CT) imaging, which can be quantified using standardized Hounsfield units. For MRI radiomics, such a unit does not exist which poses problems due to inter- and intra-scanner variability. Multiple pre-processing methods have been developed, though not all radiomics features were shown to be robust between different pre-processing approaches [57-59]. This calls for a generalized pre-processing pipeline and focus on features that are shown to be robust. Robust features and normalization methods can be achieved by applying phantom studies to account for differences between MRI acquisition protocols [60]. Tumor delineation poses another important aspect of radiomics feature extraction. Manual delineation is still generally seen as the golden standard, though a substantial inter-observer variability exists, despite international guidelines on tumor delineation [61] and it is a time consuming process. It has been shown that this inter-observer variation influences the radiomics analysis in multiple tumors [62]. Automatic segmentation methods using a deep learning neural network approach are widely developed and can be beneficial in future radiomics studies and its clinical applicability by decreasing workload on clinicians and inter-observer variability [63,64]. This is expected to lead to more robust radiomics features due to standardization of the delineation method.

Parallel to the establishment of MR signatures that are able to predict clinically significant expression of specific biomarkers, there is a need for imaging signatures that capture the level of intratumoral heterogeneity. However, it needs to be emphasized that is not yet clarified how to quantify GBM MR imaging heterogeneity and moreover how to non-invasively analyze the level of intratumoral heterogeneous expression of predictive markers, since the golden standard, single cell RNA sequencing, is missing in standard of care. By extracting radiomics features from the whole tumor and the surrounding area of edema we identified several features that are associated with intratumor heterogeneity. However, different steps could be taken to include more aspects of tumor heterogeneity. Improved performance of radiomics has been reported when features are extracted from distinct tumor areas (active tumor, necrosis and edema) separately [65,66], though this is a more labor-intensive approach which might limit its clinical applicability. In this aspect, automatic segmentation algorithms have shown to be useful for prognostic radiomics modelling [47]. Also, more biologically relevant MRI sequences such as diffusion- or perfusion-weighted MRI has been shown to outperform radiomics models based on conventional MRI [25]. These approaches should be taken into account in future studies as they will be able to encompass more features concerning intratumor heterogeneity [67] and have shown improved performance with regards to predicting prognosis and molecular markers. Ultimately, studies correlation pathological and genetic examination of multiregional biopsies towards imaging features are needed to study the value of imaging features for tumor heterogeneity.

Materials and Methods

Patient population

All patients treated by the neuro-oncology team of the Maastricht University Medical Centre (Maastricht UMC+) between January 2004 and August 2014 for a

glioblastoma (WHO grade IV) were considered for inclusion in the retrospective training cohort. Patients were excluded if no diagnostic, pre-operative MRI-images were available (minimum T1+Gadolinium and T2-weighed imaging), if survival data was unknown or no histological diagnosis was available. All patient records were reviewed considering patient and tumor characteristics, received treatments, and survival data. The external validation cohort was constructed using the same criteria on an independent dataset of patients treated in Radboud University Medical Center (Radboudumc) Nijmegen in the same time period. Both Maastricht UMC+ and Radboudumc are academic reference centers for GBM patients in the Netherlands, implying MRI-images were also obtained in hospitals that refer their patients to these academic centers. Numbers of patients used for each analysis are reported in Supplementary Table 1. The requirement for informed consent for this retrospective study was waived by the medical-ethics committee of the MUMC+ (METC 16-4-022).

Image acquisition and qualitative imaging feature assessment

Pre-operative MRI images were collected, pseudonymized and pooled in a database combining MRI images from different types and manufacturers of scanners using different imaging protocols to reflect the real-life inter-center heterogeneity (Supplementary Figure 1 and 2). A quantitative and qualitative imaging analysis pipeline was set-up (Figure 3). All diagnostic MRI-scans were analyzed by dedicated neuro-radiologists (SP, AJ, AP), blinded for outcome, and scored using the VASARI Imaging Features. A previous study conducted by the VASARI research project group showed a strong overall inter-observer agreement among six readers for the VASARI features [29]. When needed, multi-categorical and continuous VASARI features were recoded into different groups based on their clinical relevance prior to the start of analysis (Supplementary Table 5).

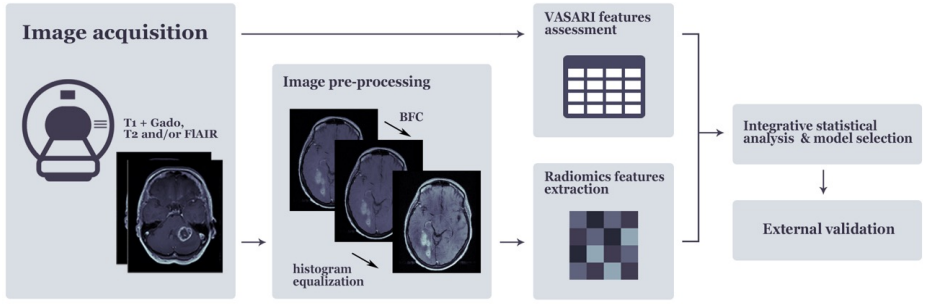

Figure 3. Quantitative and qualitative imaging analysis pipeline.

Tumor delineation, image pre-processing and extraction of radiomics features

Using Osirix Lite and MiM software (version 7.0.4), regions of interests (enhancing tumor on T1+Gadolinium images and combined tumor/edema portion on T2-weighted images) were manually delineated on all diagnostic MRI-images of the training and validation cohort, supervised by two experienced neuro-radiation oncologists (DE, IC).

Using Python 3.7 and the dedicated packages (cv2 version 4.1.0, SimpleITK version 1.2.0 and scikit-image version 0.14.2), an image pre-processing routine was developed to handle the broad variability of image acquisition and reconstruction parameters.

At first, spatial resolution of the images was normalized with respect to the image sequence (final pixels are: 0.449mm^2 and slice thickness of: 5.5mm). The mode of the pixel spacing and slice thickness distributions from the Maastricht UMC+ cohort were used as reference values for the resampling procedure to minimize the number of resampled images. A bicubic interpolation over 4x4 pixel neighborhood was used for both upsampling and downsampling. In order to correct the low frequency intensity non-uniformity, which is intrinsic for MRI images, the N4 bias field correction algorithm was used [68]. Furthermore, the histogram equalization method implemented in the scikit-image 0.15.0 package [69], was used to enhance the contrast of MRI images [70]. As the last step of the pre-processing routine, image intensities were normalized using Z-score standardization method [71]. A pre-processing routine was applied to both cohorts, where parameters (mu, sigma) for the Z-score transformation were evaluated on the training cohort and transferred to the validation cohort. Parameters used are T1 mu = 0.1904, T1 sigma = 0.2313, T2 mu = 0.2009 and T2 sigma = 0.2448.

In order to obtain the quantitative imaging features an open-source Pyradiomics 2.2.0 python package for the radiomics features extraction was utilized [72]. Using the dedicated MRI settings, features from following feature classes were extracted: First Order Statistics, Shape-based (2D and 3D), Gray Level Cooccurence Matrix (GLCM), Gray Level Run Length Matrix (GLRLM), Gray Level Size Zone Matrix (GLSZM), Gray Level Dependence Matrix (GLDM), Neighboring Gray Tone Difference Matrix (NGTDM). Along with the original features Laplacian of Gaussian (LoG)(sigma: [2.0,3.0,4.0.5.0] and Wavelet filters were activated resulting in a total of 1197 features per patient. A detailed mathematical feature description as provided by Aerts et al. 2014 [30].

Molecular markers

Archival formalin-fixed paraffin-embedded (FFPE) tissue samples were analyzed for tumor percentage by an experienced neuro-pathologist (JB). DNA was extracted from FFPE tissue using the Cobas method (Roche) and DNA concentration was quantified using Qubit Fluorometer (Life Technologies). Next-generation sequencing (NGS) was performed using the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) as previously described [73]. For the purpose of this study the data was analyzed for the presence of an IDH1 (R132H) mutation (minimum coverage 500x) which was manually checked using Integrative Genomics Viewer (IGV). EGFR amplification was assessed using SNPitty, an open-source web application for interactive B-allele frequency and copy number visualization of NGS data, by comparing the number of reads in the EGFR locus to the surrounding regions [74]. MGMT methylation status was assessed using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) as previously described [75]. In case NGS data was not available for a sample, MLPA was also used to assess IDH1 mutation status and EGFR amplification.

Statistical analysis

Statistical analysis for differences between baseline characteristics was performed using double-sided T-test for 'age at diagnosis'. Fisher's exact test was used for all other binary variables (sex, type of surgery, adjuvant treatment and molecular markers).

Overall survival (OS) was defined as the time between the initial surgical intervention after diagnosis and the date of death (confirmed by the Municipal Personal Records Database). Patients that survived were censored at the moment of the last follow-up measurement. To develop a prognostic model analysis was focused on the IDH-WT GBM samples.

OS analysis was performed using R (version 4.0.2.), employing the packages stats, survival, survminer, rms, pec and survcomp. VASARI features were tested in univariate Cox-regression analysis to determine the hazard ratio (HR) of each feature individually on the training cohort. Each feature with a p-value of ≤ 0.2 was considered for inclusion in the multivariable analysis. Resulting VASARI features were used for multivariable Cox-regression analysis with fast backward elimination (removal alpha <0.2) on the training set. Radiomics features from T1- and T2-weighted images were combined and normalized with the Z-score transformation, where coefficients evaluated on the train set were transferred to the validation set. Highly correlated features exceeding the Spearman's rank correlation of rs = 0.85 were eliminated. . Resulting radiomics features,were used for multivariable Cox-regression analysis with fast backward elimination for the training set [76] (Model 1-3) All clinical features

were entered into the Cox-regression model to develop the Clinical model on the training set.. A prognostic Index (PI) for all models developed on the training set was calculated for training and validation datasets, where the PI was defined as for each individual model For the combined models, the PI of the individual modelswas used as a feature along with the PI for the individual model it was combined with in Cox-regression analysis [77]. Similarly, for a combined clinical/VASARI/radiomics model (Model 7), VASARI PI was used as a feature along the radiomics PI and clinical PI. Next, the models were validated using multiple-step approach [78]. Calibration slope was assessed using the Log-rank (LR) test. Model's misspecification was evaluated by performing the Cox regression on the individual features of the signature in the validation dataset with offsetting the validation PI [78].

Overall model performance for discriminating survival groups was evaluated with Harrell's C-index. To display the potential discrimination between survival groups Kaplan-Meier (KM) curves were used with the threshold value based on 75th percentile of training PI's in order to identify a high-risk group using our model. Significance of the split was estimated using the LR test. In addition, predicted survival curves for each risk group were plotted. The PI is used to estimate the survival curve, which is then averaged over the entire risk-group. These curves are plotted alongside the observed KM-curves. The correlation between radiomics features and tumor volume was assessed using Spearman's rank correlation. This was investigated since previous studies have shown some radiomics features to be surrogate markers for tumor volume and not independent prognostic features [79]. Correlation between VASARI features, radiomics features and clinical features were assessed using the point-biserial correlation coefficient.

Python 3.7 was used to develop and validate the predictive models. Patients with unknown outcomes (molecular markers) were excluded from the analysis. At first, highly correlated features (rs>0.85) were eliminated, in which the feature with the lower AUC value in univariate ROC analysis was removed and resulting features were normalized using Z score on the MUMC+ cohort. Shift/scale parameters of individual features are available upon request. As the second step, the MUMC+ cohort was split randomly into train and test sets with the 70/30 ratio and label stratification. In the third step to obtain the feature importance scores, random forest model with the random-sampled initialization of hyper parameters (each iteration parameters were randomly sampled from the hyper parameter ranges: number of estimators [20,300], max depth [2,6]) was fitted for 1000 times resulting in the cumulative feature importance histogram. Based on the feature importance rank, the 20 most important features were selected for the further evaluation. In order to find the best performing model in the fourth step

Xgboost, Random Forest and Logistic regression algorithms was initialized with the random-sampling of hyper parameters (Supplementary Table 6), trained, and tested for 1000 times. In order to overcome a "lucky split bias", step 2 (the random splitting of the cohorts) followed by model testing was repeated for 10 times for the top 5 performing models from step 4, representing the cross validation technique.

Combined model was achieved by ensembling VASARI and radiomics models using averaging of VASARI and radiomics predicted probabilities. To evaluate performance of the predictive models the area under the receiver operating characteristic (ROC) curve, or AUC, was calculated. Bootstrapping technique with 100 iterations was utilized to estimate ROC AUC 95% confidence intervals on test and validation datasets.

Additionally, to visualize the ability of radiomics features of capturing the outcome heterogeneity in a univariate manner and contribute to concept of explainable radiomics, we visualized the outcome heterogeneity through selected radiomics features by plotting the distribution of feature values for each particular feature of each binary outcome. The significance of the difference in the mean values was evaluated by performing the Mann-Whitney test with Bonferroni correction.

TRIPOD statement and radiomics quality score (RQS)

To assess quality of the conducted study, a radiomics quality score (RQS) was calculated. The RQS is a checklist consisting of 16 components to assess the validity of the radiomics workflow and (external) validation of the models [19,80]. Furthermore, the checklist recommended in transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) was assessed [81].

Conclusions

In the present study, the potential of non-invasive quantitative and qualitative imaging features to predict prognosis and clinically relevant molecular markers was investigated in a real-life heterogeneous GBM patient cohort. The integrated prognostic model, including clinical and imaging features showed the most promising performance which was reproducible and most robust between both datasets. However, further improvements and larger prospective studies are needed before this model can be used in daily clinical practice. Using imaging features to predict molecular markers showed promising results in the testing set but could not be validated on the external validation set and warrants additional validation in larger GBM cohorts.

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Supplementary Materials:
Prognostic and predictive value of integrated qualitative and quantitative magnetic resonance imaging analysis in glioblastoma

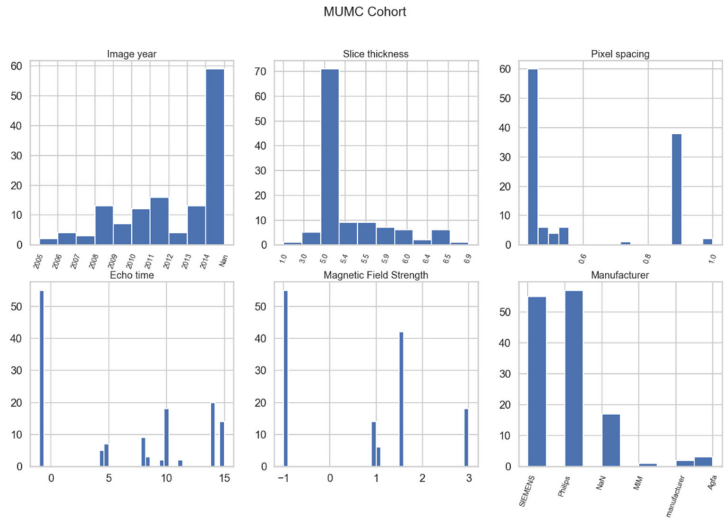

Figure S1. Imaging heterogeneity in Maastricht University Medical Center (MUMC+) cohort. Distribution of values of scanner settings in the cohort. For some images values were lost due to the pseudonymization process.

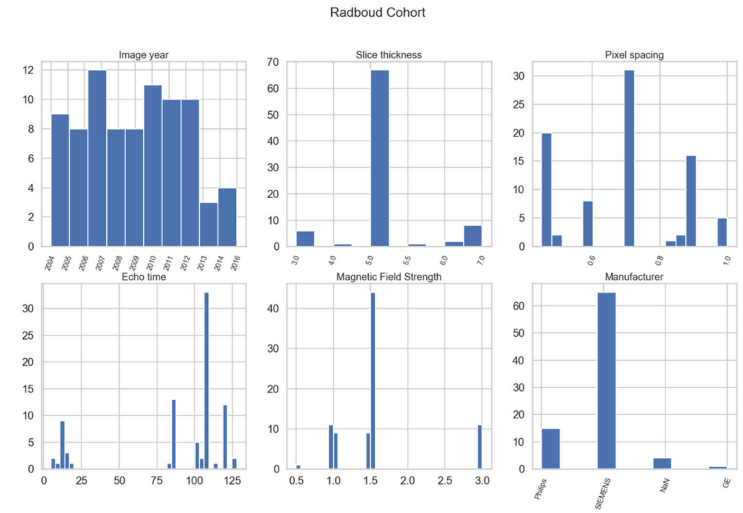

Figure S2. Imaging heterogeneity in Radboudumc cohort. Distribution of values of scanner settings in the cohort.

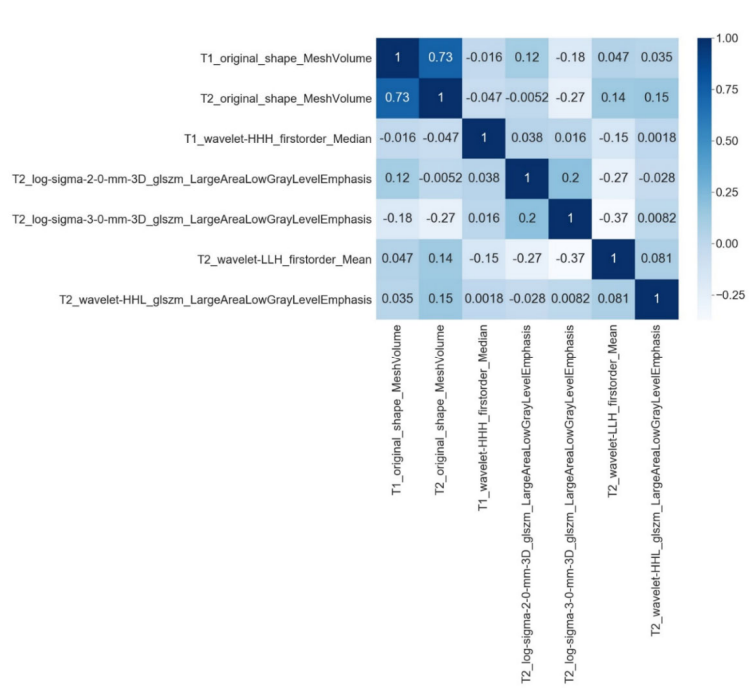

Figure S3. Correlation matrix between radiomics features and tumor volume. This was investigated since previous studies have shown some radiomics features to be surrogate markers for tumor volume and not independent prognostic factors [1]. Correlation was assessed using Spearman's rank correlation.

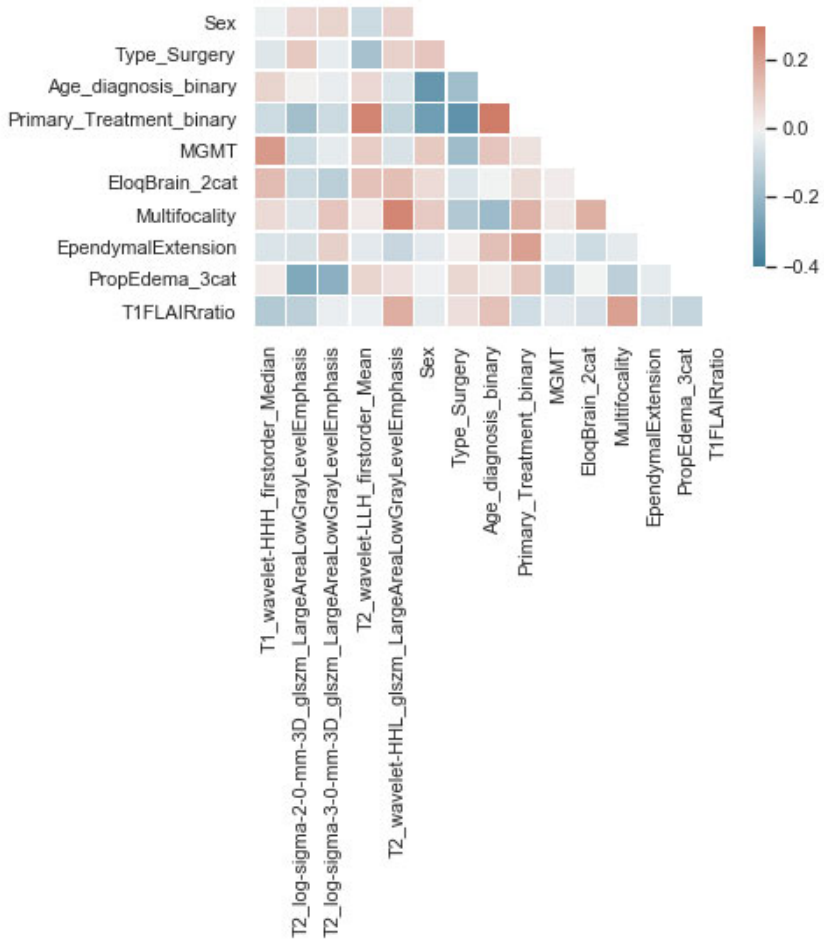

Figure S4. Correlation matrix between VASARI features, clinical features and radiomics features. Correlation was assessed using Point-Biserial Correlation Coefficient.

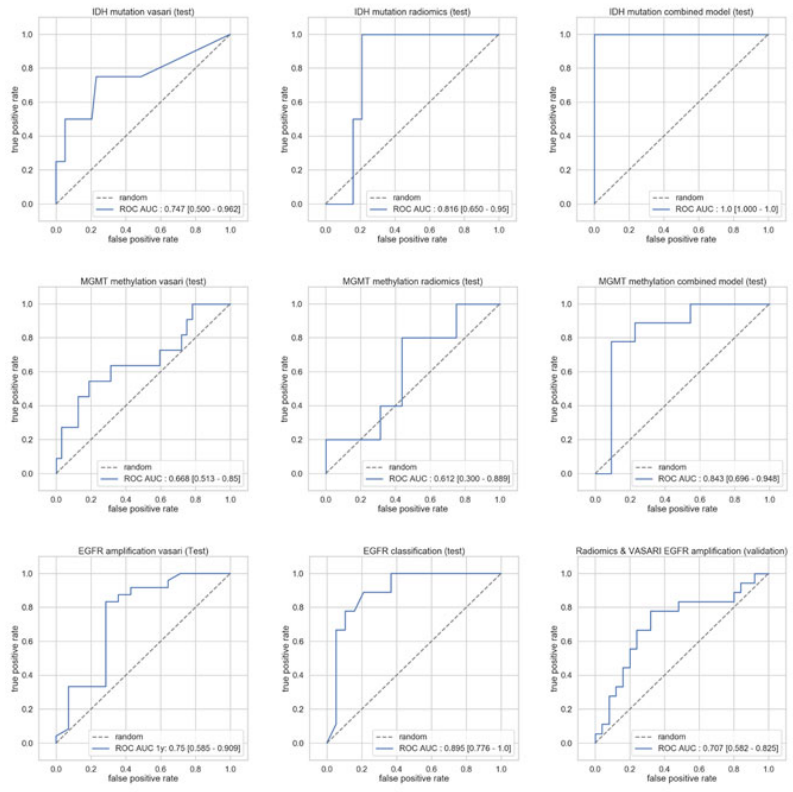

Figure S5. ROC curves for predictive models for isocitrate dehydrogenase (IDH)-mutation, methylguanine methyltransferase (MGMT)-methylation and epidermal growth factor (EGFR) amplification in the test dataset. Performance is shown for each outcome using VASARI features alone (left row), Radiomics features alone (middle row) or VASARI and Radiomics combined (right row). AUC values and 95% confidence intervals are reported.

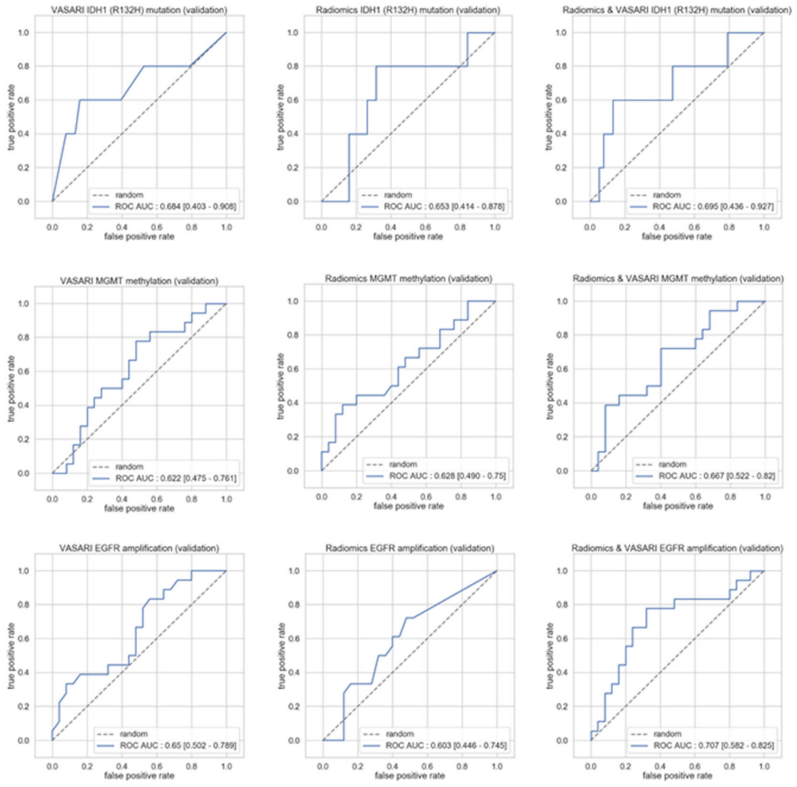

Figure S6. ROC curves for predictive models for isocitrate dehydrogenase (IDH)-mutation, methylguanine methyltransferase (MGMT)-methylation and epidermal growth factor (EGFR) amplification in the validation dataset. Performance is shown for each outcome using VASARI features alone (left row), Radiomics features alone (middle row) or VASARI and Radiomics combined (right row). AUC values and 95% confidence intervals are reported.

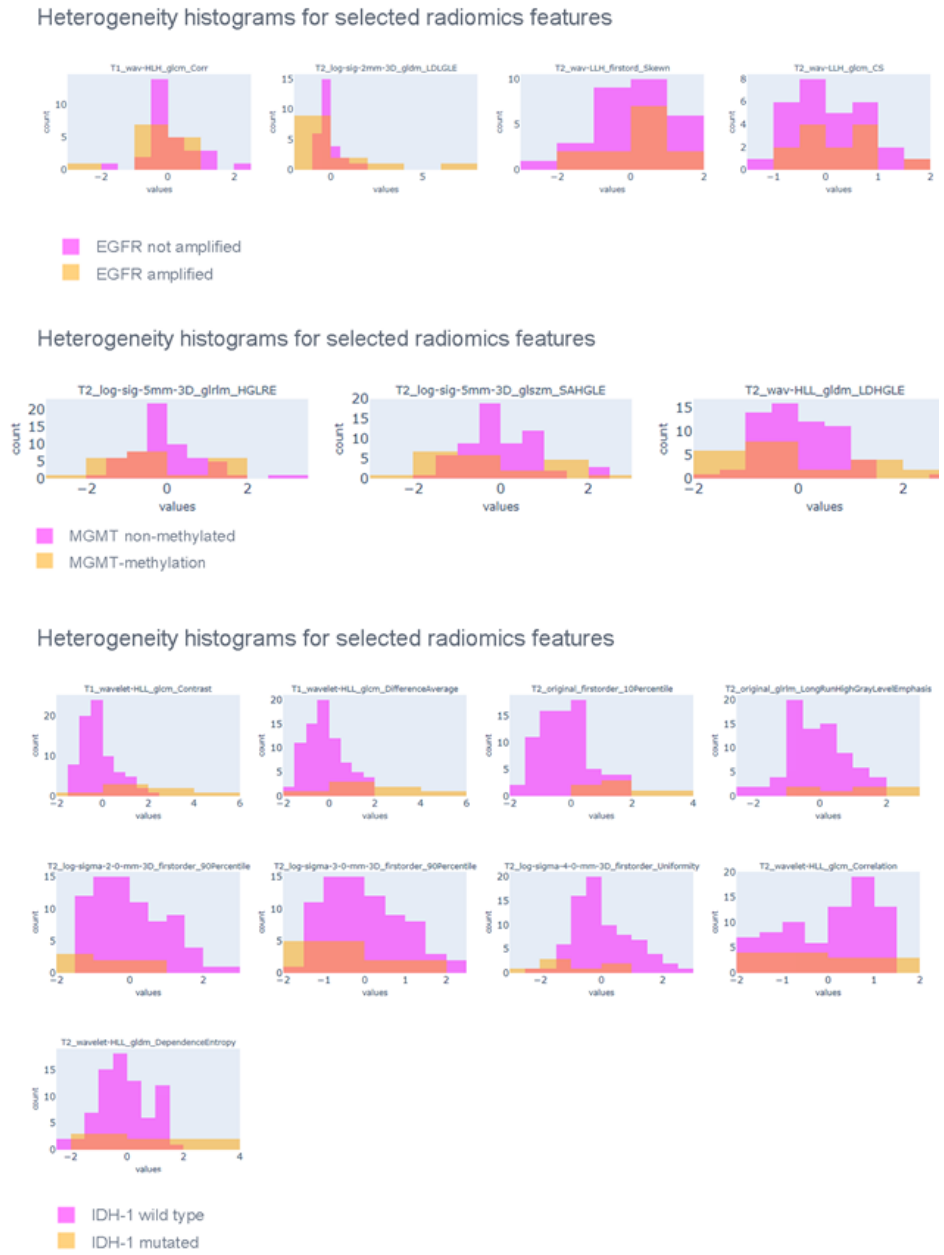

Figure S7. Heterogeneity histograms from the selected radiomics features in predictive models. To visualize the ability of radiomics features of capturing the outcome heterogeneity in a univariate manner the outcome heterogeneity is visualized

through selected radiomics features by plotting the distribution of feature values for each particular feature in the predictive models for epidermal growth factor receptor (EGFR) amplification, methylguanine methyltransferase (MGMT) methylation and isocitrate dehydrogenase (IDH)1mutation status.

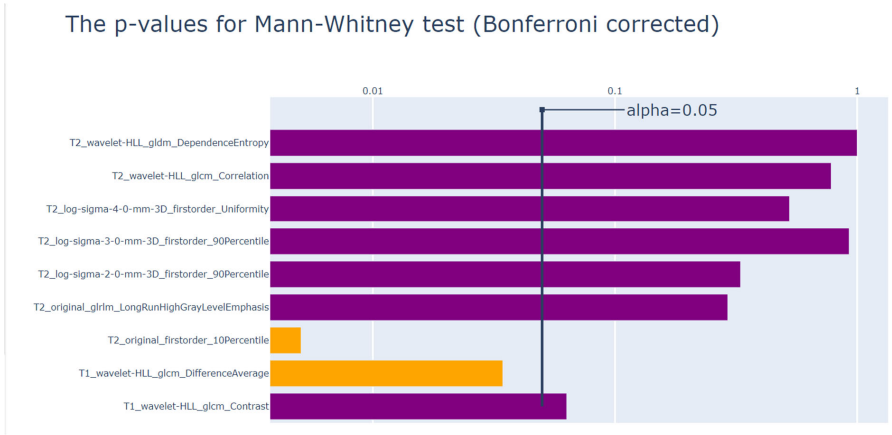

Figure S8. Mann-Whitney test for significance of histogram heterogeneity of radiomics features in predicting isocitrate dehydrogenase (IDH)1 mutation status. Significance is reported for the difference in mean values and after applying Bonferroni correction. No significance was found for histogram heterogeneity of radiomics features for epidermal growth factor receptor (EGFR) amplification or methylguanine methyltransferase (MGMT) methylation (data not shown).

Table S1. Overview of numbers of patients used for the development and validation of each mode presented in this study. Differences between numbers can be explained due to missing MR images needed for radiomics analysis or insufficient quality of images.

Outcome	VASARI[1]	Radiomics[2]	VASARI& Radiomics[3]	Clinical, VASARI&Clinical, VASARI&Radiomics[4]	VASARI& Radiomics& Clinical[5]
Overall survival isocitrate dehydrogenase (IDH)-WT GBM	Train: 129 Validation: 38	Train: 95 Validation: 38	Train: 95 Validation: 38	Train: 95 Validation: 38	Train: 95 Validation: 38
Epidermal growth factor receptor (EGFR) amplification	Train: 64 Test: 28 Validation: 44	Train: 64 Test: 28 Validation: 44	Train: 64 Test: 28 Validation: 44	N.A.	N.A.
Methylguanine methyltransferase (MGMT)-methylation	Train: 74 Test: 30 Validation: 43	Train: 74 Test: 30 Validation: 43	Train: 74 Test: 30 Validation: 43	N.A.	N.A.
IDH1-mutation status	Train: 72 Test: 30 Validation: 43	Train: 72 Test: 30 Validation: 43	Train: 72 Test: 30 Validation: 43	N.A.	N.A.

[1]For all VASARI patients T1+C and T2 and/or FLAIR MR images were available. For 20 patients in the training cohort and 5 patients in the validation cohort no FLAIR image was available and T2 was used instead for features involving FLAIR.
[2]For all Radiomics patients T1+C and T2 MR images were available. Patients for which only FLAIR and not T2 images were available were not included in this group.
[3]For VASARI&Radiomics model all patients in the Radiomics cohort were included.

[4]For Clinical model all patients in the Radiomics cohort were included.
[5]For the Combined model all patients in the Radiomics cohort were included.

Table S2. Univariate Cox-regression analysis of VASARI features for overall survival in isocitrate dehydrogenase (IDH)-wild type glioblastoma population.

VASARI feature	Overall Survival		
	HR	(95% CI)	p-value
Major axis (mm)	1.072	0.994-1.156	0.071
Major axis (median cut-off) (<6,9 vs. >6,9)	1.505	1.047-2.164	0.027
Major axis (mean cut-off) (<7,00 vs. >7,00)	1.440	1.003-2.068	0.048
Minor axis (mm)	1.044	0.935-1.165	0.442
Minor axis (mean cut-off) (<4,80 vs. >4,80)	1.318	0.919-1.890	0.134
Minor axis (median cut-off) (<4,65 vs. >4,65)	1.472	1.012-2.140	0.043
Tumor location (frontal, temporal, parietal, occipital, insular, basal ganglia, thalamus, brainstem, cerebellum, corpus callosum)			n.s.
Tumor side (right, central/bilateral, left)			n.s.
Involvement of eloquent brain (yes vs. no)	1.359	0.940-1.965	0.103
Enhancement Quality (mild/marked vs. no)	0.852	0.373-1.950	0.705
Cyst (yes vs. no)	1.949	0.946-4.015	0.070
Distribution (focal vs. non-focal)	1.494	0.877-2.547	0.140
T1/FLAIR ratio (non-expansive vs. expansive) NB : for 109 patients FLAIR was available for T1/FLAIR ratio. For the other 20 patients T2 was used instead.	0.745	0.483-1.149	0.183
Thickness of enhancing margin (thick/nodular/solid vs. thin/no enhancing margin).	1.386	0.829-2.317	0.213
Definition of enhancing margin (poorly defined vs. well defined/no enhancing margin)	1.002	0.551-1.822	0.994
Definition of non-enhancing margin (poorly defined vs. well defined/no non-enhancing margin)	1.154	0.772-1.725	0.485
Haemorrhage (yes vs. no)	0.858	0.597-1.234	0.409
Pial invasion (yes vs. no)	0.857	0.596-1.232	0.404
Subependymal extension (yes vs. no)	1.542	1.076-2.208	0.018
Cortical involvement (yes vs. no)	0.741	0.431-1.275	0.279
Deep white matter invasion (yes vs. no)	1.448	0.990-2.118	0.056
Non-contrast enhancing tumor crosses midline (yes vs. no)	1.343	0.829-2.174	0.231
Contrast-enhancing tumor crosses midline (yes vs. no)	1.291	0.763-2.187	0.342
Satellites (yes vs. no)	1.179	0.727-1.912	0.504
Proportion of contrast-enhancing tumor (≤33%, 34-66%, ≥67%)			n.s.
Proportion of non contrast-enhancing tumor (≤33%, 34-66%, ≥67%)			n.s.
Proportion of necrosis (≤33%, 34-66%, ≥67%)			n.s.
Proportion of edema (≤33%, 34-66%, ≥67%)			1 dummy variable <0.2

Table S3. TRIPOD statement assessment for this study. TRIPOD is a checklist recommended in transparent reporting of a multivariable prediction model for individual prognosis or diagnosis.

No.	Y=yes; N=no; R=referenced; NA=not applicable	Development [D]	External validation [V]	Combined Development & External validation [D+V]
1	**Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted.**			**0**
i	The words developing/development, validation/validating, incremental/added value (or synonyms) are reported in the title	N	N	N
ii	The words prediction, risk prediction, prediction model, risk models, prognostic models, prognostic indices, risk scores (or synonyms) are reported in the title	Y	Y	Y
iii	The target population is reported in the title	Y	Y	Y
iv	The outcome to be predicted is reported in the title	Y	Y	Y
2	**Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions.**			**0**
i	The objectives are reported in the abstract	Y	Y	Y
ii	Sources of data are reported in the abstract *E.g. Prospective cohort, registry data, RCT data.*	Y	Y	Y
iii	The setting is reported in the abstract *E.g. Primary care, secondary care, general population, adult care, or paediatric care. The setting should be reported for both the development and validation datasets, if applicable.*	Y	Y	Y
iv	A general definition of the study participants is reported in the abstract *E.g. patients with suspicion of certain disease, patients with a specific disease, or general eligibility criteria.*	Y	Y	Y
v	The overall sample size is reported in the abstract	N	N	N
vi	The number of events (or % outcome together with overall sample size) is reported in the abstract *If a continuous outcome was studied, score Not applicable (NA).*	N	N	N
vii	Predictors included in the final model are reported in the abstract. For validation studies of well-known models, at least the name/acronym of the validated model is reported *Broad descriptions are sufficient, e.g. 'all information from patient history and physical examination'.* *Check in the main text whether all predictors of the final model are indeed reported in the abstract.*	N	N	N
viii	The outcome is reported in the abstract	N	N	N
ix	Statistical methods are described in the abstract *For model development, at least the type of statistical model should be reported. For validation studies a quote like "model's discrimination and calibration was assessed" is considered adequate. If done, methods of updating should be reported.*	Y	Y	Y
x	Results for model discrimination are reported in the abstract *This should be reported separately for development and validation if a study includes both development and validation.*	Y	Y	Y
xi	Results for model calibration are reported in the abstract *This should be reported separately for development and validation if a study includes both development and validation.*	N	N	N
xii	Conclusions are reported in the abstract *In publications addressing both model development and validation, there is no need for separate conclusions for both; one conclusion is sufficient.*	N	N	N
3a	**Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models.**			**1**
i	The background and rationale are presented	Y	Y	Y
ii	Reference to existing models is included (or stated that there are no existing models)	Y	Y	Y
3b	**Specify the objectives, including whether the study describes the development or validation of the model or both.**			**1**
i	It is stated whether the study describes development and/or validation and/or incremental (added) value	Y	Y	Y
4a	**Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable.**			**1**
i	The study design/source of data is described *E.g. Prospectively designed, existing cohort, existing RCT, registry/medical records, case control, case series.* *This needs to be explicitly reported; reference to this information in another article alone is insufficient.*	Y	Y	Y
4b	**Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up.**			**1**
i	The starting date of accrual is reported	Y	Y	Y
ii	The end date of accrual is reported	Y	Y	Y
iii	The length of follow-up and prediction horizon/time frame are reported, if applicable *E.g. "Patients were followed from baseline for 10 years" and "10-year prediction of…"; notably for prognostic studies with long term follow-up.* *If this is not applicable for an article (i.e. diagnostic study or no follow-up), then score Not applicable (NA).*	Y	Y	Y
5a	**Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres.**			**1**
i	The study setting is reported (e.g. primary care, secondary care, general population) *E.g.: 'surgery for endometrial cancer patients' is considered to be enough information about the study setting.*	Y	Y	Y
ii	The number of centres involved is reported *If the number is not reported explicitly, but can be concluded from the name of the centre/centres, or if clearly a single centre study, score Yes.*	Y	Y	Y

iii	The geographical location (at least country) of centres involved is reported *If no geographical location is specified, but the location can be concluded from the name of the centre(s), score Yes.*	Y	Y	Y
5b	**Describe eligibility criteria for participants.**			**1**
i	In-/exclusion criteria are stated *These should explicitly be stated. Reasons for exclusion only described in a patient flow is not sufficient.*	Y	Y	Y
5c	**Give details of treatments received, if relevant.** *(i.e. notably for prognostic studies with long term follow-up)*			**1**
i	Details of any treatments received are described *This item is notably for prognostic modelling studies and is about treatment at baseline or during follow-up. The 'if relevant' judgment of treatment requires clinical knowledge and interpretation.* *If you are certain that treatment was not relevant, e.g. in some diagnostic model studies, score Not applicable.*	Y	Y	Y
6a	**Clearly define the outcome that is predicted by the prediction model, including how and when assessed.**			**1**
i	The outcome definition is clearly presented *This should be reported separately for development and validation if a publication includes both.*	Y	Y	Y
ii	It is described how outcome was assessed (including all elements of any composite, for example CVD [e.g. MI, HF, stroke]).	Y	Y	Y
iii	It is described when the outcome was assessed (time point(s) since T0)	Y	Y	Y
6b	**Report any actions to blind assessment of the outcome to be predicted.**			**1**
i	Actions to blind assessment of outcome to be predicted are reported *If it is clearly a non-issue (e.g. all-cause mortality or an outcome not requiring interpretation), score Yes. In all other instances, an explicit mention is expected.*	Y	Y	Y
7a	**Clearly define all predictors used in developing or validating the multivariable prediction model, including how and when they were measured.**			**1**
i	All predictors are reported *For development, "all predictors" refers to all predictors that potentially could have been included in the 'final' model (including those considered in any univariable analyses).* *For validation, "all predictors" means the predictors in the model being evaluated.*	Y	Y	Y
ii	Predictor definitions are clearly presented	Y	Y	Y
iii	It is clearly described how the predictors were measured	Y	Y	Y
iv	It is clearly described when the predictors were measured	Y	Y	Y
7b	**Report any actions to blind assessment of predictors for the outcome and other predictors.**			**0**
i	It is clearly described whether predictor assessments were blinded for outcome *For predictors for which it is clearly a non-issue (e.g. automatic blood pressure measurement, age, sex) and for instances where the predictors were clearly assessed before outcome assessment, score Yes. For all other predictors an explicit mention is expected.*	Y	Y	Y
ii	It is clearly described whether predictor assessments were blinded for the other predictors	N	N	N
8	**Explain how the study size was arrived at.**			**1**
i	It is explained how the study size was arrived at *Is there any mention of sample size, e.g. whether this was done on statistical grounds or practical/logistical grounds (e.g. an existing study cohort or data set of a RCT was used)?*	Y	Y	Y
9	**Describe how missing data were handled (e.g., complete-case analysis, single imputation, multiple imputation) with details of any imputation method.**			**1**
i	The method for handling missing data (predictors and outcome) is mentioned *E.g. Complete case (explicit mention that individuals with missing values have been excluded), single imputation, multiple imputation, mean/median imputation.* *If there is no missing data, there should be an explicit mention that there is no missing data for all predictors and outcome. If so, score Yes.* *If it is unclear whether there is missing data (from e.g. the reported methods or results), score No.* *If it is clear there is missing data, but the method for handling missing data is unclear, score No.*	Y	Y	Y
ii	If missing data were imputed, details of the software used are given *When under 9i explicit mentioning of no missing data, complete case analysis or no imputation applied, score Not applicable.*	NA	NA	NA
iii	If missing data were imputed, a description of which variables were included in the imputation procedure is given *When under 9i explicit mentioning of no missing data, complete case analysis or no imputation applied, score Not applicable.*	NA	NA	NA
iv	If multiple imputation was used, the number of imputations is reported *When under 9i explicit mentioning of no missing data, complete case analysis or no imputation applied, score Not applicable.*	NA	NA	NA
10a	**Describe how predictors were handled in the analyses.**			**1**
i	For continuous predictors it is described whether they were modelled as linear, nonlinear (type of transformation specified) or categorized *A general statement is sufficient, no need to describe this for each predictor separately.* *If no continuous predictors were reported, score Not applicable.*	NA	Not applicable	NA
ii	For categorical or categorized predictors, the cut-points were reported *If no categorical or categorized predictors were reported, score Not applicable.*	Y	Not applicable	Y
iii	For categorized predictors the method to choose the cut-points was clearly described *If no categorized predictors, score Not applicable.*	Y	Not applicable	Y
10b	**Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation.**			**0**

i	The type of statistical model is reported *E.g. Logistic, Cox, other regression model (e.g. Weibull, ordinal), other statistical modelling (e.g. neural network)*	Y	Not applicable	Y
ii	The approach used for predictor selection <u>before</u> modelling is described *'Before modelling' means before any univariable or multivariable analysis of predictor-outcome associations.* *If no predictor selection before modelling is done, score Not applicable.* *If it is unclear whether predictor selection before modelling is done, score No.* *If it is clear there was predictor selection before modelling but the method was not described, score No.*	Y	Not applicable	Y
iii	The approach used for predictor selection <u>during</u> modelling is described *E.g. Univariable analysis, stepwise selection, bootstrap, Lasso.* *'During modelling' includes both univariable or multivariable analysis of predictor-outcome associations.* *If no predictor selection during modelling is done (so-called full model approach), score Not applicable.* *If it is unclear whether predictor selection during modelling is done, score No.* *If it is clear there was predictor selection during modelling but the method was not described, score No.*	Y	Not applicable	Y
iv	Testing of interaction terms is described *If it is explicitly mentioned that interaction terms were not addressed in the prediction model, score Yes.* *If interaction terms were included in the prediction model, but the testing is not described, score No.*	N	Not applicable	N
v	Testing of the proportionality of hazards in survival models is described *If no proportional hazard model is used, score Not applicable.*	Y	Not applicable	Y
vi	Internal validation is reported *E.g. Bootstrapping, cross validation, split sample.* *If the use of internal validation is clearly a non-issue (e.g. in case of very large data sets), score Yes. For all other situations an explicit mention is expected.*	Y	Not applicable	Y
10c	**For validation, describe how the predictions were calculated.**			**1**
i.	It is described how predictions for individuals (in the validation set) were obtained from the model being validated *E.g. Using the original reported model coefficients with or without the intercept, and/or using updated or refitted model coefficients, or using a nomogram, spreadsheet or web calculator.*	Not applicable	Y	Y
10d	**Specify all measures used to assess model performance and, if relevant, to compare multiple models.** *These should be described in methods section of the paper (item 16 addresses the reporting of the results for model performance).*			**0**
i	Measures for model discrimination are described *E.g. C-index / area under the ROC curve.*	Y	Y	Y
ii	Measures for model calibration are described *E.g. calibration plot, calibration slope or intercept, calibration table, Hosmer Lemeshow test, O/E ratio.*	N	N	N
iii	Other performance measures are described *E.g. R2, Brier score, predictive values, sensitivity, specificity, AUC difference, decision curve analysis, net reclassification improvement, integrated discrimination improvement, AIC.*	N	N	N
10e	**Describe any model updating (e.g., recalibration) arising from the validation, if done.**			**Not applicable**
i	A description of model-updating is given *E.g. Intercept recalibration, regression coefficient recalibration, refitting the whole model, adding a new predictor* *If updating was done, it should be clear which updating method was applied to score Yes.* *If it is not explicitly mentioned that updating was applied in the study, score this item as 'Not applicable'.*	Not applicable	NA	NA
11	**Provide details on how risk groups were created, if done.** *If risk groups were not created, score this item as Yes.*			**1**
i	If risk groups were created, risk group boundaries (risk thresholds) are specified *Score this item separately for development and validation if a study includes both development and validation.* *If risk groups were not created, score this item as not applicable.*	Y	Y	Y
12	**For validation, identify any differences from the development data in setting, eligibility criteria, outcome and predictors.**			**1**
i	Differences or similarities in <u>definitions</u> with the development study are described *Mentioning of any differences in all four (setting, eligibility criteria, predictors and outcome) is required to score Yes.* *If it is explicitly mentioned that there were no differences in setting, eligibility criteria, predictors and outcomes, score Yes.*	Not applicable	Y	Y
13a	**Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful.**			**1**
i	The flow of participants is reported	NA	NA	NA
ii	The number of participants with and without the outcome are reported *If outcomes are continuous, score Not applicable.*	Y	Y	Y
iii	A summary of follow-up time is presented *This notably applies to prognosis studies and diagnostic studies with follow-up as diagnostic outcome.* *If this is not applicable for an article (i.e. diagnostic study or no follow-up), then score Not applicable.*	Y	Y	Y
13b	**Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome.**			**1**
i	Basic demographics are reported	Y	Y	Y

ii	Summary information is provided for all predictors included in the final developed/validated model	Y	Y	Y
iii	The number of participants with missing data for predictors is reported	Y	Y	Y
iv	The number of participants with missing data for the outcome is reported	Y	Y	Y
13c	**For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome).**			**1**
i	Demographic characteristics (at least age and gender) of the validation study participants are reported along with those of the original development study	Not applicable	Y	Y
ii	Distributions of predictors in the model of the validation study participants are reported along with those of the original development study	Not applicable	Y	Y
iii	Outcomes of the validation study participants are reported along with those of the original development study	Not applicable	Y	Y
14a	**Specify the number of participants and outcome events in each analysis.**			**1**
i	The number of participants in each analysis (e.g. in the analysis of each model if more than one model is developed) is specified	Y	Not applicable	Y
ii	The number of outcome events in each analysis is specified (e.g. in the analysis of each model if more than one model is developed) *If outcomes are continuous, score Not applicable.*	Y	Not applicable	Y
14b	**If done, report the unadjusted association between each candidate predictor and outcome.**			**0**
i	The unadjusted associations between each predictor and outcome are reported *If any univariable analysis is mentioned in the methods but not in the results, score No.* *If nothing on univariable analysis (in methods or results) is reported, score this item as Not applicable.*	N	Not applicable	N
15a	**Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at a given time point).**			**1**
i	The regression coefficient (or a derivative such as hazard ratio, odds ratio, risk ratio) for each predictor in the model is reported	Y	Not applicable	Y
ii	The intercept or the cumulative baseline hazard (or baseline survival) for at least one time point is reported	Y	Not applicable	Y
15b	**Explain how to use the prediction model.**			**0**
i	An explanation (e.g. a simplified scoring rule, chart, nomogram of the model, reference to online calculator, or worked example) is provided to explain how to use the model for individualised predictions.	N	Not applicable	N
16	**Report performance measures (with confidence intervals) for the prediction model.** *These should be described in results section of the paper (item 10 addresses the reporting of the methods for model performance).*			**0**
i	A discrimination measure is presented *E.g. C-index / area under the ROC curve.*	Y	Y	Y
ii	The confidence interval (or standard error) of the discrimination measure is presented	Y	Y	Y
iii	Measures for model calibration are described *E.g. calibration plot, calibration slope or intercept, calibration table, Hosmer Lemeshow test, O/E ratio.*	N	N	N
iv	Other model performance measures are presented *E.g. R2, Brier score, predictive values, sensitivity, specificity, AUC difference, decision curve analysis, net reclassification improvement, integrated discrimination improvement, AIC.*	N	N	N
17	**If done, report the results from any model updating (i.e., model specification, model performance, recalibration).** *If updating was not done, score this TRIPOD item as 'Not applicable'.*			**Not applicable**
0	Model updating was done *If "No", then answer 17i-17v with "Not applicable"*	Not applicable	N	N
i	The updated regression coefficients for each predictor in the model are reported *If model updating was described as 'not needed', score Yes.*	Not applicable	NA	NA
ii	The updated intercept or cumulative baseline hazard or baseline survival (for at least one time point) is reported *If model updating was described as 'not needed', score Yes.*	Not applicable	NA	NA
iii	The discrimination of the updated model is reported	Not applicable	NA	NA
iv	The confidence interval (or standard error) of the discrimination measure of the updated model is reported	Not applicable	NA	NA
v	The calibration of the updated model is reported	Not applicable	NA	NA
18	**Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data).**			**1**
i	Limitations of the study are discussed *Stating any limitation is sufficient.*	Y	Y	Y
19a	**For validation, discuss the results with reference to performance in the development data, and any other validation data.**			**1**
i	Comparison of results to reported performance in development studies and/or other validation studies is given	Not applicable	Y	Y
19b	**Give an overall interpretation of the results considering objectives, limitations, results from similar studies and other relevant evidence.**			**1**
i	An overall interpretation of the results is given	Y	Y	Y
20	**Discuss the potential clinical use of the model and implications for future research.**			**1**
i	The potential clinical use is discussed *E.g. an explicit description of the context in which the prediction model is to be used (e.g. to identify high risk groups to help direct treatment, or to triage patients for referral to subsequent care).*	Y	Y	Y
ii	Implications for future research are discussed *E.g. a description of what the next stage of investigation of the prediction model should be, such as "We suggest further external validation".*	Y	Y	Y

21	**Provide information about the availability of supplementary resources, such as study protocol, web calculator, and data sets.**			**1**
i	Information about supplementary resources is provided	Y	Y	Y
22	**Give the source of funding and the role of the funders for the present study.**			**1**
i	The source of funding is reported or there is explicit mention that there was no external funding involved	Y	Y	Y
ii	The role of funders is reported or there is explicit mention that there was no external funding	Y	Y	Y
	Number of applicable TRIPOD items			**35**
	Number of TRIPOD items adhered			**27**
	OVERALL adherence to TRIPOD			**77%**

Table S4. Radiomics Quality Score (RQS) assessment for this study. The RQS is a checklist consisting of 16 components to assess the validity of the radiomics workflow and (external) validation of the models.

No.	Radiomics aspects	Maximum points to be scored	Score for this study
1	Image protocol quality - well-documented image protocols (for example, contrast, slice thickness, energy, etc.) and/or usage of public image protocols allow reproducibility/replicability	+ 1 (if protocols are well-documented) + 1 (if public protocol is used)	0
2	Multiple segmentations - possible actions are: segmentation by different physicians/algorithms/software, perturbing segmentations by (random) noise, segmentation at different breathing cycles. Analyse feature robustness to segmentation variabilities	1	0
3	Phantom study on all scanners - detect inter-scanner differences and vendor-dependent features. Analyse feature robustness to these sources of variability	1	0
4	Imaging at multiple time points - collect images of individuals at additional time points. Analyse feature robustness to temporal variabilities (for example, organ movement, organ expansion/shrinkage)	1	0
5	Feature reduction or adjustment for multiple testing - decreases the risk of overfitting. Overfitting is inevitable if the number of features exceeds the number of samples. Consider feature robustness when selecting features	− 3 (if neither measure is implemented) + 3 (if either measure is implemented)	3
6	Multivariable analysis with non radiomics features (for example, EGFR mutation) - is expected to provide a more holistic model. Permits correlating/inferencing between radiomics and non radiomics features	1	1
7	Detect and discuss biological correlates - demonstration of phenotypic differences (possibly associated with underlying gene–protein expression patterns) deepens understanding of radiomics and biology	1	1
8	Cut-off analyses - determine risk groups by either the median, a previously published cut-off or report a continuous risk variable. Reduces the risk of reporting overly optimistic results	1	1
9	Discrimination statistics - report discrimination statistics (for example, C-statistic, ROC curve, AUC) and their statistical significance (for example, p-values, confidence intervals). One can also apply resampling method (for example, bootstrapping, cross-validation)	+ 1 (if a discrimination statistic and its statistical significance are reported) + 1 (if a resampling method technique is also applied)	2
10	Calibration statistics - report calibration statistics (for example, Calibration-in-the-large/slope, calibration plots) and their statistical significance (for example, *P*-values, confidence intervals). One can also apply resampling method (for example, bootstrapping, cross-validation)	+ 1 (if a calibration statistic and its statistical significance are reported) + 1 (if a resampling method technique is also applied)	1
11	Prospective study registered in a trial database - provides the highest level of evidence supporting the clinical validity and usefulness of the radiomics biomarker	+ 7 (for prospective validation of a radiomics signature in an appropriate trial)	0
12	Validation - the validation is performed without retraining and without adaptation of the cut-off value, provides crucial information with regard to credible clinical performance	- 5 (if validation is missing) + 2 (if validation is based on a dataset from the same institute) + 3 (if validation is based on a dataset from another institute) + 4 (if validation is based on two datasets from two distinct institutes) + 4 (if the study validates a previously published signature) + 5 (if validation is based on three or more datasets from distinct institutes)	3

13	Comparison to 'gold standard' - assess the extent to which the model agrees with/is superior to the current 'gold standard' method (for example, TNM-staging for survival prediction). This comparison shows the added value of radiomics	2	2
14	Potential clinical utility - report on the current and potential application of the model in a clinical setting (for example, decision curve analysis).	2	2
15	Cost-effectiveness analysis - report on the cost-effectiveness of the clinical application (for example, QALYs generated)	1	0
16	Open science and data - make code and data publicly available. Open science facilitates knowledge transfer and reproducibility of the study	+ 1 (if scans are open source) + 1 (if region of interest segmentations are open source) + 1 (if code is open source) + 1 (if radiomics features are calculated on a set of representative ROIs and the calculated features and representative ROIs are open source)	1
	Total score:	36	17

Table S5. Definitions of VASARI features as used in this study. Adapted from *Wangaryattawanich et al [2].*

VASARI feature	Definition
Major axis (mm)	The longest diameter of the tumor which is based upon measurement of the FLAIR (or T2) abnormality on a single axial image that demonstrates the largest cross-sectional area.
Major axis (median cut-off) (<6,9 vs. >6,9)	Binary classification of major axis above or below median value as determined in the training cohort.
Major axis (mean cut-off) (<7,00 vs. >7,00)	Binary classification of major axis above or below mean value as determined in the training cohort.
Minor axis (mm)	The diameter of the FLAIR (or T2) abnormality which is perpendicular to the longest diameter. The measurement is performed on a single asxial image that demonstrates the largest cross-sectional area.
Minor axis (mean cut-off) (<4,80 vs. >4,80)	Binary classification of minor axis above or below median value as determined in the training cohort.
Minor axis (median cut-off) (<4,65 vs. >4,65)	Binary classification of minor axis above or below mean value as determined in the training cohort.
Tumor location (frontal, temporal, parietal, occipital, insular, basal ganglia, thalamus, brainstem, cerebellum, corpus callosum)	Location of (largest portion of) the tumor, (including both contrast-enhancing (CET) or non-contrast-enhancing tumor (nCET).
Tumor side (right, central/bilateral, left)	Side of lesion epicentre irrespective of whether lesion crosses into the contralateral hemisphere.
Involvement of eloquent brain (yes vs. no)	Presence of tumor involvement in the eloquent cortex (speech motor, speech receptive, motor or vision).
Enhancement Quality (mild/marked vs. no)	Qualitative degree of contrast enhancement (significantly higher signal on postcontrast T1W images compared with precontrast T1W images).
Cyst (yes vs. no)	Well-defined, rouded regions of very bright T2W signal and low T1W signal (matching CSF signal) with tin, regular, smoot, non-enhancing or regularly enhancing walls, possibly with thin, regular, internal septations.
Distribution (focal vs. non-focal)	Non-focal tumors include tumors of which at least one region of tumor (either CET or nCET) is not contiguous with the dominant lesion and outside the region of signal abnormality surrounding the dominant mass.
T1/FLAIR ratio (non-expansive vs. expansive)	Gross composition in the overall lesion size between precontrast T1 and FLAIR (or T2) in the same plan. Expansive (T1=FLAIR) or non-expansive (T1<FLAIR).
Thickness of enhancing margin (thick/nodular/solid vs. thin/no enhancing margin).	The thickness of the enhancing margin of the tumor. Not applicable if there is no contrast enhancement. Thick is considered ≥3mm, thin ≤3mm.
Definition of enhancing margin (poorly defined vs. well defined/no enhancing margin)	The definition of the outside enhancing margin of the tumor. Not applicable if there is no contrast enhancement.
Definition of non-enhancing margin (poorly defined vs. well defined/no non-enhancing margin)	The definition of the outside margin of the non-enhancing margin of the tumor.
Haemorrhage (yes vs. no)	Intrinsic haemorrhage anywhere within the tumor matrix (any foci of low signal on T2WI or high signal on T1WI).
Pial invasion (yes vs. no)	Enhancement of the overlying pia in continuity with enhancing or non-enhancing margin.
Subpendymal extension (yes vs. no)	Invasion of any adjacent ependymal surface in continuity with enhancing or non-enhancing tumor.
Cortical involvement (yes vs. no)	Non-enhancing or enhancing tumor extending to the cortical mantle or cortex.
Deep white matter invasion (yes vs. no)	Enhancing or non-enhancing tumor extending into the internal capsule, corpus callosum or brainstem.
Non-contrast enhancing tumor crosses midline (yes vs. no)	nCET crosses into the contralateral hemisphere through white matter commissures.
Contrast-enhancing tumor crosses midline (yes vs. no)	Enhancing tissue crosses into contralateral hemisphere through with matter commissures.
Satellites (yes vs. no)	An area of enhancement within the region of signal abnormality surrounding the dominant lesion but not continuous with the major enhancing tumor mass.
Proportion of contrast-enhancing tumor (≤33%, 34-66%, ≥67%)	Visually estimated proportion of enhancing component of the entire tumor.
Proportion of non contrast-enhancing tumor (≤33%, 34-66%, ≥67%)	Visually estimated proportion of non-enhancing component of the entire tumor.
Proportion of necrosis (≤33%, 34-66%, ≥67%)	Visually estimated proportion of necrosis to the entire tumor (non-enhancing region with high signal on T2W images and low on T1W images).
Proportion of edema (≤33%, 34-66%, ≥67%)	Visually estimated proportion of edema relative to the entire tumor mass (CET+nCET+necrosis).

Table 6. Tuned hyperparameters used for development of predictive models for isocitrate dehydrogenase (IDH)-mutation, methylguanine methyltransferase (MGMT)-methylation and epidermal growth factor receptor (EGFR) amplification.

Model	Hyper-parameter values
Logistic regression (Scikit-learn version 0.21.3)	number of features: [2:15] Penalty: [L1,L2]
Random Forest (Scikit-learn version 0.21.3)	number of features: [2:15] max depth: [2:7] number of estimators: [50:300]
XGBoost (xgboost version 0.90)	number of features: [2:15] max depth: [2:7] number of estimators: [50:300], learning rate: [0.1, 0.15, 0.2, 0.25, 0.3, 0.01, 0.03, 0.09, 0.001, 0.003]

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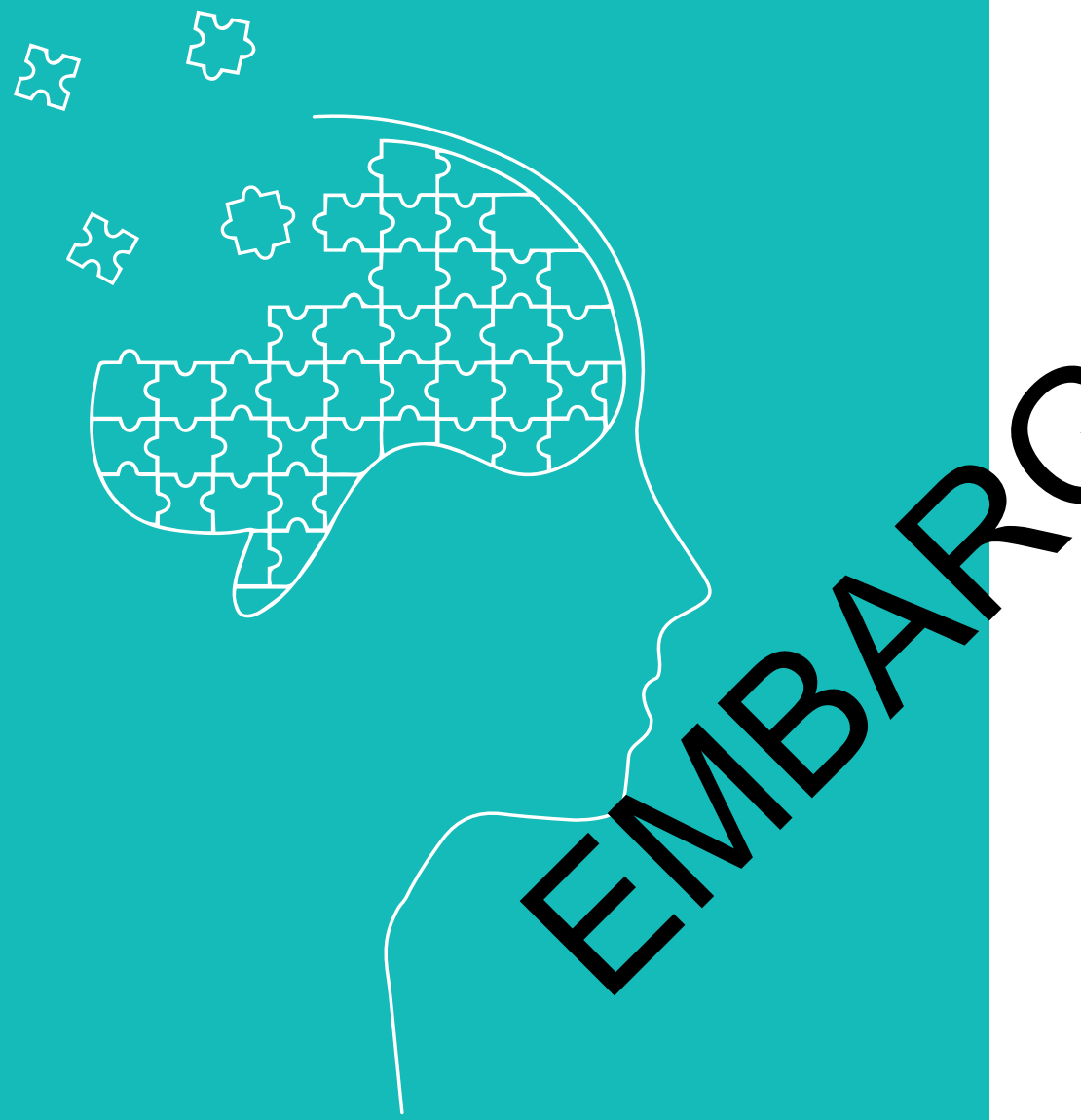

Chapter 6

Integrating tissue architecture in the genomic landscape of newly diagnosed glioblastoma
A preliminary report

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Chapter 7

General Discussion

Glioblastoma (GBM) is the most aggressive primary brain tumor that, despite intensive multimodal treatment, remains incurable to this date. Tumor heterogeneity is widely accepted as one of the major determinants of treatment failure and the abysmal prognosis of GBM patients [1]. Other factors include the poor penetration of anti-cancer drug through the blood-brain-barrier [2], the immune-suppressive micro-environment in GBM [3] and its diffuse invasion of healthy brain parenchyma [4]. Decades of research to identify novel treatment options have thus far not led to substantial improvements in the standard-of-care treatment and ultimately prognosis of GBM patients.

The research presented in this thesis explores different methods to study tumor heterogeneity in GBM, in both the pre-clinical and clinical context. This thesis aimed to identify novel methods to integrate tumor heterogeneity in future translational and clinical studies to ultimately aid in improving patient outcome in GBM.

The first part of this thesis, Chapter 2 and 3, focusses on the application of patient-derived cancer organoids in cancer research and the development and validation of a patient-derived GBM organoid model. The second part, including Chapter 4 and 5, studies non-invasive diagnostic modalities, including routinely available magnetic resonance images (MRI) to predict prognosis and molecular tumor markers in GBM. Finally, Chapter 6 is directed at integrating tumor genetics and tissue architecture to study implications and composition of the heterogeneity of the GBM immune micro-environment at the single-cell level.

Patient-derived GBM organoids

In traditional pre-clinical cancer studies, tumor heterogeneity is an aspect that is not represented [5]. Since intratumoral heterogeneity is widely appreciated as a major determinant in treatment effectiveness [1,6-8], novel methods have been developed to incorporate intratumoral heterogeneity in pre-clinical research models. In previous research, across the entire field of cancer research, patient-derived cancer organoids have been proposed as a model to achieve this. As cancer organoids are a stem-cell based model that retain self-renewal properties and are able to undergo multilineage differentiation they are able to maintain intratumoral heterogeneity and propagate indefinitely [9,10]. Cancer organoids are developed using cancer cells derived from primary patient material embedded in a basement-membrane extract (i.e. Matrigel) and cultured under serum-free, stem-cell stimulating conditions [11] allowing for both the maintenance and multilinear differentiation of cancer stem cells (CSCs). Three-dimensional cell culture models have shown to be superior to traditional 2D cell lines in several ways. For example, 3D cultures have shown to be more resistant towards radio- and chemotherapy, possibly due to cell-matrix interactions, nuclear organization and chromatin structure [12]. In traditional 2D cell culture, components from the extracellular matrix are lacking and cell-cell interactions are less representative, which are important determinants of cell differentiation, function and proliferation [13].

Previous research has shown that cancer organoids resemble the parental tumor on a genetic, transcriptomic and phenotypic level in multiple types of cancer [14-17] and have multiple promising applications in cancer research [11,18].

Whilst early research regarding patient-derived cancer organoids has already thoroughly studied their resemblance to the primary tumor, Chapter 2 reviews the clinical utility and the potential of cancer organoids to predict treatment response in patients. The ultimate promise of cancer organoids would be to act as functional patient avatars that can be used to select the most effective treatment option for each individual patient, therefore aiding in personalized cancer treatment.

Thus far, patient-derived cancer organoids have been established for a large set of solid tumors but most extensively for abdominal cancers such as gastro-intestinal, liver and pancreatic cancer.

In Chapter 2, we reviewed 60 studies that subjected organoids to treatment and correlated this to patient outcome or potential biomarkers. Most studies were focused on comparing organoid treatment response to molecular alterations in the parental tumor, however only few studies actually compared organoid treatment response to clinical outcomes of the corresponding patients. Moreover, studies that were able to compare organoid treatment response to clinical outcomes mostly included case-reports. Overall, organoid treatment response correlated well to the expected response based on molecular alterations in the tumor and corresponding organoid. Cancer organoids were even used, though again in case-reports, to prospectively select a patients' treatment with good clinical outcome [19]. Perhaps even more interesting is the observation that in some cases, cancer organoids responded differently than predicted by driver mutations present in the tumor and corresponding organoid [15], which we also observed in our own GBM organoid study as described in Chapter 3. This might implicate that cancer organoids are a more complex model to test drug response that, apart from the driver mutations, also incorporates the interference of (sub)clonal mutations and mutations in parallel pathways due to the maintenance of intratumoral (genetic) heterogeneity. However,

whether this organoid treatment response also correlates with clinical treatment response in a superior way when compared to the expected treatment response based on driver mutations is subject to future research.

Thus far, few studies were able to make actual comparisons between the organoid treatment response and treatment response of the corresponding patient. The largest study to date (N=80) reported a sensitivity and specificity of 78% and 92% respectively regarding the prediction of clinical response towards neoadjuvant chemoradiation in rectal cancer patients [20]. Recently, a pooled analysis was conducted reporting a sensitivity of 81% (95% CI 69-89%) and a specificity of 75% (95% CI 0.64-0.82) for discriminating patients with a clinical response through cancer-organoid based screening [21].

All in all, this shows the potential of cancer organoids to act as predictors for clinical treatment response. It should be noted that quantitative data about the actual predictive value is scarce and larger, preferably co-clinical, trials are needed to advance its clinical implementation. Additionally, it should be considered that additional factors influencing treatment response such as the tumor micro-environment are currently not incorporated in cancer organoid models.

Intratumoral heterogeneity has also been an important subject of study in GBM and has shown to be a major determinant of treatment resistance and tumor relapse [1]. Intratumoral heterogeneity is highly underestimated since whole tumor single-cell analysis of GBM is hardly ever conducted and it is therefore not possible to determine the exact extent of intratumoral heterogeneity. Moreover, due to its infiltrative growth a complete tumor resection can never be achieved. Recently, GBM cells have been shown to exist in multiple plastic cellular states that are influenced by genetics and the tumor micro-environment [22]. Single-cell RNA sequencing identified cells that exist in states that recapitulate neural-progenitor-like (NPC-like), oligodendrocyte-progenitor-like (OPC-like), astrocyte-like (AC-like) and mesenchymal-like (MES-like) states. It has been shown that specific genetic drivers, such as epidermal growth factor receptor (EGFR), platelet derived growth factor receptor alpha (PDGFRA) and cyclin-dependent kinase 4 (CDK4) influence the frequency of those states. Also, micro-environmental factors such as hypoxia, glycolysis and immune cells are associated with specific cellular states. We hypothesised that by culturing primary GBM cells in organoids, these different cellular states are also recapitulated in the organoid by maintaining genetic drivers influencing these states as well as mimicking an oxygen gradient and thereby creating hypoxic areas due to its three-dimensional growth pattern.

In vitro models reflecting intratumoral heterogeneity are in urgent need. The currently available traditional GBM cell lines, used in GBM research, have been shown to poorly represent primary human gliomas. Such cell lines are traditionally cultured in serum-based medium and it has been shown these cells lose their self-renewing capabilities, have no ability to differentiate and are neither clonogenic nor tumorigenic [23]. Also, gene expression profiles of cell lines vary greatly from the parental tissue and cluster more strongly with each other than the parental tissue, suggesting similar adaptions occur in these cells due to culture conditions [24,25].

Since patient-derived cancer organoids have proven to be a genetically and phenotypically stable replicate of the parental tumor we set out to develop a patient-derived GBM organoid (PGO) model. Chapter 3 describes the development and validation of our GBM organoid model and shows its potential for drug screening purposes and studying treatment resistance mechanisms.

We confirmed that PGOs are a genetically stable culture model for GBM that maintains key features of GBM *in vitro*. Whole exome sequencing of PGOs and their parental tumor showed a large concordance between somatic variants and copy number variations for most of the patients. The occurrence of discrepancies between some tumor samples and PGO has also been observed in other cancer organoid studies. Possible explanations include lower tumor purity in the tumor sample compared to the PGO [16] as well as the existence of a sampling bias due to using a small sample of tumor tissue to derive cancer organoids, therefore presenting an underrepresentation of all subclones present in the tumor.

PGOs were also shown to preserve genetic and phenotypic heterogeneity based on single-cell karyotype sequencing and multiplex immunohistochemistry, both showing the existence of multiple subclones of tumor cells within the organoid. We also observed that the distribution of different subclones within the PGO largely corresponded with the parental tumor.

Additionally, PGOs were used for drug screening of standard-of-care as well as novel treatment options in GBM. As a proof of concept we sought out to compare pre- and post-treatment PGOs with temozolomide (TMZ). PGOs were treated with human equivalent dosages of TMZ for five consecutive days, after which they were dissociated and used to regrow as PGOs after which this cycle was repeated. We hypothesised this approach would stimulate the outgrowth of TMZ-resistant cells within the PGO. RNA-sequencing of pre- and post-treatment PGOs identified upregulation of the JUN kinase pathway, therefore a potential contributor to TMZ

resistance. High expression of JUN was indeed associated with a shorter progression-free survival in the TCGA database. Our study confirms previous findings [26] that inhibition of JUN-kinase sensitizes cells to TMZ as we observed a synergistic effect of TMZ and JUN-kinase inhibition in PGOs.

All in all, Chapter 3 describes the development of a potentially clinically relevant and heterogeneous GBM organoid biobank that have the potential to be used in future research including the discovery of novel treatment options as well as identifying treatment resistance mechanisms to improve the currently available options.

As is also mentioned in Chapter 2, several aspects currently limit the (pre-)clinical implementation of GBM organoids. This includes the lack of standardization in organoid culture and derivation. The methods of tumor processing prior to organoid derivation differs between studies, i.e. tumor tissue might be dissociated into single-cells or minced into tumor fragments, before embedding cells into a basement membrane extract (BME). Similarly, the type of BME and its composition largely differs between different organoid studies. The most commonly used BME compositions (for example Matrigel and Cultrex) include animal-derived components with up to 50% batch-to-batch variation in protein content [27]. Another important aspect to consider is the differences between the composition of the BME used and the extracellular matrix (ECM) in which the tumor normally resides. In example, the brain ECM greatly differs from the composition of Matrigel as it contains less structural proteins such as collagen and more proteoglycans which influence neural cell behaviour by regulating cell adhesion and neurite outgrowth [28]. In order to standardize the BME used in organoid studies, synthetically generated matrices are currently being developed of which the composition can be strictly regulated [29] and possibly also adjusted to the specific ECM that is required for a specific tumor type. These synthetic matrices are however more expensive and labour intensive and have shown a lower culture efficiency of organoids [30]. The need for standardization also applies to the different compositions of the culture medium used in organoid culture. Medium composition has shown to greatly influence cell differentiation and organoid growth [31]. For optimal organoid growth, medium composition should be tailored on the specific tumor micro-environment (TME) of each specific cancer type as well as contain standardized medium components to improve reproducibility [30]. Optimal medium composition can also help to overcome the overgrowth of healthy tissue cells in cancer organoids, such as observed in non-small cell lung cancer [32]. Approaches by adapting culture medium by for example adding Nutlin to select for TP53+ cells have been used to overcome this [33]. Caution on this should be held as this will enable selection for specific tumor cells which will reduce the representation of intratumoral heterogeneity. An optimal method to prevent the outgrowth of healthy tissue cells has not yet been developed and will be greatly dependent on this specific type of cancer and its molecular alterations.

Currently, attempts to standardize organoid derivation have been published and will aid into a more uniform approach [34]. It should however be kept in mind that different research questions might ask for specific methods of organoid culture and derivation (for example the maintenance of tissue architecture when not dissociating into single-cell suspensions).

Even though studies have already shown the representation of intratumoral heterogeneity in cancer organoids in a superior way when compared to traditional cell culture models, it has to be acknowledged that cancer organoids do not form an exact copy of the parental tumor. A major limiting factor in this includes that derivation of cancer in general and GBM organoids more specifically from a single tumor biopsy. This precludes a sampling bias into the organoid model and an underrepresentation of all (sub)clones, including metastatic tumor sites, present in the tumor, a phenomenon that we have also observed in our own GBM organoid model (Chapter 3). Multiregional biopsies have been suggested as a manner to improve on this, as well as using mixed cell populations from, in example, liquid biopsies that might give a better representation of the complete intratumoral heterogeneity. Previous studies have shown that different regions within GBM harbour different signatures [35] which has to be acknowledged when using PGOs as GBM stem cells from different regions might also have different potentials for cellular differentiation. Also, the effects of the tumor micro-environment on the differentiation state of GBM cells should also be acknowledged.

Also, GBM organoids are not yet able to capture temporal heterogeneity in time. As collecting a new tumor sample from a recurrent cancer is not always feasible it would be interesting to study whether cancer organoids acquire similar resistance mechanisms under selection of treatment pressure as would happen *in vivo* and therefore create representative recurrent cancer/GBM avatars.

Another complicating factor in this is the highly infiltrative nature of GBM into the healthy brain tissue. This implies that complete radical resection of the tumor is impossible to achieve and therefore this infiltrative subset of GBM cells, which are expected to exhibit different cell characteristics due to their infiltrative properties, cannot be accounted for in tumor analysis and derivation of primary cell culture models [36]. Specific models that mimick glioma cell invasion in cerebral organoids

have been been developed to overcome this issue which showed destruction of cerebral tissue by glioma cells, mirroring human disease pathology [37]. It would be interesting to derive PGOs specifically from cells taken from the invasive edge of the tumor and study differences in tumor growth and invasiveness when compared to PGOs derived from different regions of the tumor.

Apart from intratumoral heterogeneity, the tumor micro-environment (TME) is getting increasingly more attention for its role in cancer development and progression and also treatment resistance. In the most commonly used cancer organoid studies, the other cells present in the TME, such as endothelial cells, stromal cells and immune cells, are largely lacking. Attempts to include stromal cells have already been made and has shown to influence the organoid response to treatment. In example, co-culture of liver cancer organoids with cancer-associated fibroblasts increased resistance towards treatment [38]. Additionally, more advanced models such as tumor-on-a-chip microfluidic models which mimics a running blood stream [39] have been developed to include the effect of tumor vasculature. Due to the emergence of immunotherapy inclusion of components of the immune micro-environment gained increasing attention [40]. Immune cells were either retained by preserving the original tissue architecture [41,42], co-culturing lymph node cells [43] or peripheral blood mononuclear cells [44] with lung cancer organoids. Interestingly, autologous tumor-reactive T-cells could be induced which specifically kills tumor organoids whilst leaving the matched healthy airway organoids unaffected [44]. These models have been used to study T-cell response and efficacy of immune checkpoint inhibitors or CAR-T cell therapy and will aid in the advancement of immunotherapy in cancer treatment.

In conclusion, patient-derived organoids, including our GBM patient-derived organoids, provide a novel platform to study tumor biology, heterogeneity and treatment response. At this moment they provide a superior pre-clinical model when compared to traditional cell lines due to their improved representation of tumor genetics, intratumoral heterogeneity and maintenance of cell-cell interactions. This makes organoids well suited models to study effectiveness of anti-cancer treatments and to study adaptations that occur in cancer cells due to this treatment. In their current state however, GBM organoids are not an exact copy of the parental tumor and limitations as mentioned before should be taken into account.

Newer developments, by including components of the tumor micro-environment or using a BME that more closely resembles the brain ECM, improve the resemblance to *in vivo* GBMs but should be tailored to the research questions a specific study tries to answer as more complex models come with additional challenges. Despite the many advantages that PGOs provide the previously mentioned limitations should be addressed in future studies.

Additionally, topic for further research is whether these GBM organoids develop the same resistance mechanisms to currently available treatments compared to the in vivo GBM. And thus questions remain whether these GBM organoids could be used as co-clinical avatars to aid in selecting treatment options or monitor tumor adaptation leading to treatment resistance in personalized medicine. If so, co-clinical trials using cancer organoids can optimize patient stratification in clinical trials which will increase its chances for success and advance progression of novel treatment options from clinical trials into daily clinical practice.

Non-invasive GBM testing

Despite the established importance of inter- and intratumoral heterogeneity in GBM it has not been incorporated into daily clinical practice. As single cell analysis of multi-regional and multi-site biopsies, to identify intratumoral heterogeneity in patients is not feasible, non-invasive diagnostic methods are needed to comprehend this. Chapter 4 reviews the available non-invasive diagnostic methods available for GBM patients that could aid in incorporating tumor heterogeneity in the clinical setting.

Multiple imaging techniques are available to study GBM and could aid in identifying aspects of intratumoral heterogeneity by visualization of the entire tumor. Magnetic resonance imaging (MRI) can be used for both quantitative and qualitative analysis of GBM. The Visually Accessible Rembrandt Images (VASARI) project enlisted the most important qualitative imaging features in a standardized way [45]. Associations between these imaging features and patient prognosis, molecular GBM subtype and molecular makers have been identified but do not seem strong enough in order to be used in clinical decision making [45]. Additionally, newer machine-learning based techniques such as radiomics, provide a quantitative texture analysis on individual voxels and therefore could provide insight into intratumoral heterogeneity [46,47]. Machine-learning based artificial intelligence is the next step in using such techniques in GBM research. Deep learning models have been developed for automatic segmentation of the tumor [48], classification of brain tumors [49] as well as differentiation pseudoprogression from actual tumor progression [50]. Even though such techniques have a high potential in the future of GBM diagnostics they are not ready for actual clinical implementation. The large heterogeneity in GBM and also in imaging acquisition parameters are still challenging in developing universally applicable models, also given the fact that various studies use different data and defining criteria which complicates the comparison of different algorithms [51].

Associations between standard uptake values (SUVs) of amino-acid positron emission tomography (AA-PET) and molecular markers have also been identified. Interestingly, specific PET tracers can also be used to identify aspects of the tumor micro-environment such as tumor hypoxia using F-FMISO-PET [52] or F-HX4-PET [53]. However, these tracers are not widely available and the most commonly used FDG-PET is not useful due to its high background uptake in the normal brain tissue.

Liquid biopsies provide another non-invasive diagnostic modality to study cancer and include different types of biomarkers including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), tumor-educated platelets (TEPs), extracellular vesicles (EVs), protein metabolites and micro RNA (miRNA). Both ctDNA and CTCs have shown to be scarce in GBM and highly sensitive techniques are needed to study them [54]. ctDNA has however successfully been used to identify molecular alterations present in the tumor [55] but has not been feasible for daily clinical practice yet. TEPs seem to be more promising as they are much more abundantly present in serum of patients and can be easily isolated [56]. TEPs were successfully used to diagnose GBM based on specific RNA profiles as well as identify the presence of the endothelial growth factor receptor (EGFR) variant III mutation liquid [57]. Similarly, EVs and miRNAs seem to hold specific tumor-related information [54]but have so far not made it beyond the research setting. Despite these findings regarding liquid biopsies, the specific expertise and equipment needed for isolation and analysis and current lack of validated biomarkers are important aspects that withhold it from further development into clinical implementation, which are obstacles that are not easy to overcome.

From all non-invasive diagnostic methods, discussed in Chapter 4, which could potentially have a role in studying intratumoral heterogeneity in clinical diagnostics, MR-imaging is the most widely available modality in GBM patients. Therefore, in Chapter 5 we set out to test the potential usage of qualitative and quantitative MRI analysis to predict patient prognosis and clinically relevant molecular tumor markers.

The study described in Chapter 5 combined the qualitative VASARI features with quantitative radiomics features of diagnostic, pre-operative MR-images in GBM. By combining clinical features with VASARI and radiomics features we developed a clinically relevant model (C-index 0.723) which was also robust in the external validation cohort (C-index 0.730). Using this model we could identify a high- and low-risk group with significantly different overall survival (p-value <0.001). Even though stronger prognostic models based on imaging features have been developed, ours was able to hold up in external validation, which is commonly lacking in similar studies.

Using machine learning algorithms we developed a combined quantitative and qualitative imaging analysis model to predict clinically relevant molecular markers in GBM. Despite initial promising predictive values in our test cohort, none of the models were able to achieve clinically relevant area-under-the curve values in the external validation set.

It could be argued that the observed prognostic and predictive value of the developed imaging models is still too low for clinical implementation. Especially the predictive value of these models for molecular markers does not provide enough accuracy to be clinically useful and have an impact on clinical practice.

Even though non-invasive techniques to predict patient prognosis, molecular markers or identify intratumoral heterogeneity has been widely studied, this has not yet had implications for daily clinical practice. In order for imaging models to be clinically relevant more standardization in acquisition of MR-images, tumor delineation and feature extraction and analysis is needed. MRI acquisition parameters greatly differ between different scanners. Attempts at standardization using different methods such as histogram matching, rescaling signal intensity and deep learning have been made but no clear consensus has been reached on the optimal method to deal with this problem [58]. Inter-observer delineation variability is another aspect which influences the reproducibility of quantitative imaging features [59]. Automated delineation approaches, using deep learning, have been developed to achieve a more standardized and reproducible tumor delineation. Such approaches have shown to generate accurate GBM contours and also outperform handcrafted delineation in predicting overall survival using radiomics features [60]. Specialised MRI techniques, such as diffusion weighted imaging, perfusion weighted imaging and magnetic resonance spectroscopy also show promise in GBM radiomics as they reflect underlying tissue physiology and already contain quantified measures that are more reproducible [61]. Questions still remain however, on whether this imaging heterogeneity actually reflects molecular heterogeneity and whether the resolution that can be achieved is sufficient to improve existing prognostic and predictive methods.

In general, prognostic and predictive models need large datasets to be developed and subsequent large-scale external validation to prove its reproducibility despite of the limitations mentioned in this thesis. Current studies mostly depend on retrospective data analysis. To truly assess the clinical utility of non-invasive predictive models, prospective clinical trials are needed but not before the previously mentioned limitations have been addressed.

Multiplex spatial analysis of the GBM micro-environment

The previous chapters mainly focus on the heterogeneity of the tumor cell compartment in GBM. However, the complete architecture of the GBM micro-environment is far more complex beyond the tumor cells alone. Especially the immune micro-environment has gained a lot of interest with the emergence of different types of immunotherapy which, so far, has not been successful in GBM trials [62-64]. Chapter 6 describes the first findings of our study regarding multiplex single-cell spatial analysis of the GBM micro-environment. In the past, most single-cell studies comprised of RNA-sequencing [22,65], which lacks spatial information on the interaction between different cell types in GBM. More recently, spatial analysis has gained more attention to further improve understanding of the GBM micro-environment.

The preliminary report in this thesis describes differences in composition of the GBM micro-environment between different survival groups, different MGMT-methylation status and alterations in the EGFR gene. The relevance of our first findings will have to be explored further in additional studies including a more comprehensive overview of different subtypes of lymphoid cells. Importantly, we will also investigate the interaction between different tumor cell subtypes and components of the immune micro-environment. Furthermore, we will perform the same analysis in different genetic backgrounds in an attempt to identify subgroups of patients for specific therapeutic targets.

In conclusion, the work in this thesis demonstrated novel methods to study tumor heterogeneity in GBM in the pre-clinical and clinical setting. Patient-derived GBM organoids were proven to be a stable and representative model, maintaining intratumoral heterogeneity, which can be used to study treatment response and resistance mechanisms. Additionally, we studied the use of routinely available MR-images for quantitative and qualitative analysis and were able to develop a relevant prognostic model. Ultimately, further development and optimization of these strategies are needed to increase their clinical relevance and utility to work towards personalized medicine and subsequently a better patient outcome in GBM.

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Addenda

Summary

Summary

Glioblastoma (GBM) is the most aggressive type of primary brain cancer in adults which poses multiple clinical challenges. At present, standard-of-care treatment consist of neurosurgical resection followed by an intensive schedule of chemotherapy and radiotherapy. This treatment schedule aims to prolong a patient's life as GBM is considered to be an incurable cancer, which inevitably recurs with limited treatment options available at recurrence. Importantly, the current standard-of-care has remained unchanged over the past 15 years, despite multiple clinical trial efforts, which emphasises the large clinical need for new treatment options.

Several factors can be attributed to the lack of success of clinical trials that have been conducted in GBM research. GBM is characterized by its diffuse growth pattern throughout the healthy brain parenchyma, which extends beyond the macroscopic tumor borders that can be visualized by current imaging modalities. This implies that a complete resection of the tumor can never be achieved and radiotherapy is limited by normal tissue toxicity. Also, the location of GBM inside the brain parenchyma provides several challenges. The blood-brain-barrier (BBB) is very efficient in isolating the brain from the systemic circulation, preventing novel treatment options such as monoclonal antibodies from adequate penetration into the tumor. In addition, the unique, brain specific immune micro-environment and microglia/macrophage oriented immunosuppressive environment in GBM can explain the lack of success of T-cell oriented immune checkpoint inhibitors which have seen great success in other solid cancers.

Additionally, the presence of tumor heterogeneity is widely accepted to be on the major determinants of treatment failure and poor prognosis seen in GBM patients.

Tumor heterogeneity implies there are major differences in tumor characteristics between patients (intertumoral heterogeneity) as well as the occurrence of multiple different subclones within one patient (intratumoral heterogeneity) each with different biological and molecular characteristics, ultimately leading to differences in tumor growth and treatment response. Tumor heterogeneity can also adapt over time due to changes on the genetic level, in example under the influence of factors in the tumor micro-environment such as hypoxia. Similarly, treatment-induced genetic alterations and changes of the tumor micro-environment can occur which changes GBM biology over time.

This thesis focusses on the tumor heterogeneity in GBM and explores novel methods to study tumor heterogeneity in GBM. In pre-clinical and translational cancer research, patient-derived cancer organoids have been developed as a model which maintains tumor heterogeneity. Therefore, patient-derived cancer organoids have an improved resemblance of the actual tumor when compared to traditional cell line models.

Patient-derived cancer organoids are stem-cell based three-dimensional cell culture models which retain self-renewal properties and are able to undergo multilineage differentiation, therefore maintaining intratumoral heterogeneity.

Chapter 2 describes the previous efforts that have been made on patient-derived cancer organoids throughout all different types of cancers. This chapter focuses on how these cancer organoids are being used to predict treatment response and how they can aid in selecting the most optimal treatment strategy. We describe 60 studies in which cancer organoids have been developed for a wide variety of solid cancers in which most studies use cancer organoids for drug screenings. In a few studies, the drug response of the organoids was directly compared to the actual clinical treatment response. These studies did show promising results on their ability to predict whether a patient will show a response to a specific treatment. However, these studies are still very limited and larger trials, as well as standardization of culture protocols and measurement of drug response is needed before such approaches can actually be moved towards clinical implementation. Importantly other aspects which influence treatment response such as the tumor micro-environment should be accounted for as well in such models to improve its resemblance of the actual tumor and therefore its predictive value.

In Chapter 3 we set out to develop a patient-derived GBM organoid (PGO) model to study tumor heterogeneity in GBM. PGOs were developed by culturing GBM cells acquired from surgical resection in an extracellular matrix. We showed that these PGOs retain genetic mutations that were present in the original tumor and also maintained intratumoral heterogeneity on the single-cell level. PGOs were also suitable models to test the response towards chemotherapy (temozolomide) and radiotherapy. Interestingly, by comparing gene expression levels in PGOs before and after chemotherapy we were also able to identify the JNK pathway as a possible actionable target and determinant of sensitivity towards temozolomide. By treating PGOs with a combination of temozolomide and a small molecule JNK pathway inhibitor we showed an improved treatment response of the combination treatment

providing additional rationale for future studies on JNK inhibition in the treatment of GBM.

Besides pre-clinical approaches to improve understanding about tumor heterogeneity in GBM, novel approaches on using clinical data are also being developed. A lot of attention has been given towards utilizing novel approaches in imaging modalities such as magnetic resonance imaging (MRI) which are routinely acquired in GBM patients. Besides qualitative imaging features, such as tumor size or contrast enhancement, MR images contain more information beyond what can be observed. Computational approaches, commonly called radiomics, have been developed to extract quantitative imaging features on textures, shapes and intensities. Data gathered from this type of analysis can be used in artificial intelligence and deep learning models to develop prognostic and predictive models when combined with clinical patient data.

Chapter 4 describes non-invasive diagnostic modalities that are currently available for GBM. Non-invasive diagnostic modalities such as nuclear imaging, MRI and liquid biopsies are possible strategies to acquire information on tumor characteristics. Detection levels of circulating tumor cells or other circulating biomarkers in both the blood and cerebrospinal fluid are low and the lack of validated biomarkers as well as the necessary expertise and equipment for such analysis are major obstacles for the clinical implementation of liquid biopsies. Specific positon-emission tomography (PET) tracers have been studied as biomarkers for specific molecular characteristics of the tumor but are also not widely available.

As MRI is the most widely used diagnostic modality this holds the greatest promise in non-invasive diagnostic methods. Associations between imaging features and patient prognosis as well as molecular markers have been made in multiple studies. However, the large heterogeneity between studies on this subject impedes a direct translation towards the clinic.

In Chapter 5 we set out to develop a prognostic and predictive model using integrated quantitative and qualitative imaging analysis in GBM. This study showed that combining clinical data with quantitative and qualitative MRI imaging features resulted in the most optimal prognostic model for overall survival which could be replicated in an external validation cohort. Using these features to predict molecular alterations present in the tumor was promising after development but this could not be reproduced in an external validation cohort in a clinically relevant manner.

The previously described efforts mainly focus on cancer cells and their role in tumor heterogeneity and treatment response. Whilst, cancer cells and their biological and molecular characteristics are of course a major determinant of treatment response and patient outcome, the tumor micro-environment has gained a lot of attention in cancer research. With the emergence of immune checkpoint inhibitors and its major success in solid cancers, such approaches have also been studies in GBM but have so far only given disappointing results in clinical trials. Chapter 6 describes the first results of our multiplex single-cell immunohistochemistry analysis on a patient cohort of newly diagnosed GBM. This will provide the opportunity to study the interaction between cancer and immune cells and also thoroughly characterize the immune cells which are present in the tumor. This study will attempt to improve understanding of the immune micro-environment on the single-cell level and to identify potential strategies to improve success of immunotherapy in GBM.

In summary, this thesis describes approach to study tumor heterogeneity in GBM in both the pre-clinical and clinical setting. We showed that PGOs are promising novel GBM models that maintain intratumoral heterogeneity. The work in this thesis provides opportunities for future research on PGOs in GBM such as incorporating components of the tumor micro-environment. Also we show the possible application of computation approaches such as radiomics to develop prognostic and predictive models. Nonetheless, both techniques require standardization, optimization and further validation before they can be translated towards clinical implementation and actually influence GBM patient care.

Addenda

Samenvatting

A

Samenvatting

Het glioblastoom (GBM) is de meest agressieve vorm van hersenkanker in volwassenen. Momenteel bestaat de standaardbehandeling hiervoor uit neurochirurgische verwijdering van de tumor gevolgd door een intensief schema met chemotherapie en radiotherapie. Dit behandelschema heeft als doel om het leven van een patiënt te verlengen aangezien het GBM een ziekte is die niet genezen kan worden. Het is belangrijk om te vermelden dat de huidige standaardbehandeling al meer dan 15 jaar onveranderd is gebleven. Dit maakt duidelijk hoe groot de noodzaak is tot het vinden van nieuwe behandelopties voor patiënten met een GBM.

Er zijn verschillende factoren te noemen die er toe hebben geleid dat alle klinische studies naar nieuwe geneesmiddelen geen succes hebben gehad. Het GBM groeit diffuus door het gezonde hersenweefsel heen en dit is uitgebreider dan we met beeldvormende technieken kunnen zien. Als gevolg hiervan is het onmogelijk om een volledige chirurgische verwijdering van de tumor uit te voeren en is de bestralingsdosis die gegeven kan worden gelimiteerd door de toxiciteit op het gezonde hersenweefsel. Daarnaast zorgt de locatie van het GBM in de hersenen ook voor extra uitdagingen. De bloed-hersen-barriere (BBB) is een efficiënt mechanisme van het lichaam om de hersenen af te sluiten van de systemische circulatie. Hierdoor kunnen nieuwe geneesmiddelen, zoals monoklonale antilichamen, niet of moeilijker binnendringen in de tumor. Daarnaast is er sprake van een unieke tumor micro-omgeving waarbij er bijvoorbeeld meer microglia en macrofagen aanwezig zijn in vergelijking met andere soorten kanker. Dit kan verklaren waarom immuuntherapie, welke gericht is op T-cellen, niet effectief is gebleken in het GBM terwijl het in andere vormen van kanker veelbelovend is.

Daarnaast is de aanwezigheid van tumor heterogeniteit een belangrijke factor in het falen van behandelingen en de daaruit voortkomende slechte prognose van GBM patiënten.

Tumor heterogeniteit betekent dat er grote verschillen bestaan in tumor karakteristieken tussen patiënten (intertumorale heterogeniteit) maar ook dat er verschillen bestaan tussen groepen tumorcellen binnen eenzelfde patiënt (intratumorale heterogeniteit) met elk andere biologische en moleculaire eigenschappen. Dit leidt uiteindelijk tot verschillen in tumor groei en respons op behandeling. Deze tumor heterogeniteit kan ook veranderen door de tijd, bijvoorbeeld door omgevingsfactoren in de tumor zoals een laag zuurstofgehalte. Daarnaast kan ook de gegeven behandeling, zoals chemotherapie, bepaalde veranderingen induceren in de tumor waardoor deze minder goed reageert op ingezette behandeling.

Deze thesis is gericht op de tumor heterogeniteit in GBM en onderzoekt nieuwe methoden om deze tumor heterogeniteit te bestuderen. In preklinisch en translationeel onderzoek zijn patiënt-specifieke tumor organoïden ontwikkeld als een model om deze tumor heterogeniteit na te bootsen. Deze onderzoeken hebben laten zien dat tumor organoïden een betere afspiegeling zijn van de tumor dan traditionele cellijnen.

Patiënt-specifieke tumor organoïden zijn driedimensionele celkweek modellen ontwikkeld vanuit stamcellen. Deze organoïden behouden daardoor de mogelijkheid tot vernieuwing en differentiatie, wat uiteindelijk leidt tot behoud van intratumorale heterogeniteit.

Hoofdstuk 2 beschrijft de onderzoeken die gedaan zijn op het gebied van tumor organoïden in verschillende soorten kanker. Dit hoofdstuk richt zich specifiek op hoe deze tumor organoïden gebruikt worden om de respons op behandeling te voorspellen en hoe organoïden kunnen helpen bij het selecteren van de beste behandeloptie. We beschrijven 60 studies waarin tumor organoïden met name gebruikt worden voor het screenen naar mogelijke geschikte medicijnen. In een paar studies wordt de respons op de behandeling in de organoïd direct vergeleken met de klinische respons van de patiënt. Deze studies laten veelbelovende resultaten zien als het gaat om de mogelijkheid van tumor organoïden om te voorspellen of een patiënt wel of niet op een behandeling zal reageren. Hierbij moet wel opgemerkt worden dat dit slechts een paar studies betreft en er grotere onderzoeken nodig zijn, waarin ook kweekprotocollen en manieren om behandelrespons te meten gestandaardiseerd moeten worden, voordat dit in de kliniek geïmplementeerd kan worden. Daarnaast is het belangrijk dat ook andere aspecten die van invloed zijn op de respons op een behandeling, zoals de tumor micro-omgeving, mee te nemen om een nog accurater model van de originele tumor te krijgen.

In Hoofdstuk 3 hebben we ons eigen patiënt-specifieke GBM organoïd (PGO) model ontwikkeld om tumor heterogeniteit in het GBM te bestuderen. PGOs werden gemaakt door het kweken van GBM cellen, direct afkomstig uit de patiënt, in een extracellulaire matrix. In dit hoofdstuk laten we zien dat PGOs genetische afwijkingen die in de originele tumor aanwezig waren behouden. Daarnaast was er ook behoud van intratumorale heterogeniteit in de PGOs. PGOs bleken ook geschikt als model om de respons op chemotherapie (temozolomide) en radiotherapie te testen. Door het

vergelijkingen van gen-expressie voor en na behandeling met temozolomide hebben we de 'JNK pathway' geïdentificeerd als een mogelijke factor die gevoeligheid voor temozolomide beïnvloed. Om dit te onderzoeken hebben we PGOs behandeld met temozolomide en een JNK blokker waarbij we een verhoogde gevoeligheid van de PGOs zagen als er een combinatiebehandeling gegeven werd.

Naast preklinisch onderzoek gericht op een beter begrip van de tumor heterogeniteit in GBM worden er ook nieuwe mogelijkheden met klinische data ontwikkeld. Er is veel aandacht gekomen voor geavanceerde analyses van MRI beelden, een beeldvormende techniek welke veel gebruikt word bij GBM patiënten. Uit MRI beelden kunnen kwalitatieve eigenschappen, zoals grootte van de tumor of mate van contrastopname, bepaald worden. Daarnaast kunnen er ook kwantitatieve eigenschappen middels computer gestuurde analyse, genaamd radiomics, bepaald worden welke informatie geven over texturen, vormen en intensiteiten. Door de vele data die hiermee verkregen wordt te analyseren met kunstmatige intelligentie kunnen er prognostische en predictieve modellen ontwikkeld worden.

Hoofdstuk 4 beschrijft niet-invasieve diagnostische technieken die momenteel ontwikkeld zijn voor GBM. Deze technieken bestaan onder andere uit nucleaire beeldvorming, MRI en vloeibare biopten (liquid biopsy) waarmee informatie over tumorkarakteristieken verkregen kan worden.

Vloeibare biopten betreft het afnemen van bijvoorbeeld bloed of hersenvocht voor moleculaire analyse. Voor het GBM wordt de toepassing hiervan bemoeilijkt doordat er doorgaans lage concentraties circulerende tumorcellen of andere biomarkers gevonden worden. Daarnaast is de expertise en apparatuur welke nodig is voor deze analyses niet op veel plaatsen beschikbaar. Binnen de nucleaire geneeskunde zijn specifieke tracers voor PET-scans ontwikkeld voor het bestuderen van specifieke moleculaire eigenschappen van de tumor, maar ook deze zijn niet op veel plekken beschikbaar.

Aangezien MRI de meest gebruikte diagnostische methode is voor het GBM zit hier ook de meeste potentie. In meerdere studies zijn er associaties gemaakt tussen eigenschappen van MRI beelden, prognose van patiënten en moleculaire markers. Er bestaat echter grote heterogeniteit tussen deze studies waardoor deze nog niet geleidt hebben tot implementatie hiervan in de dagelijkse praktijk.

In Hoofdstuk 5 hebben we een prognostisch en predictief model ontwikkeld op basis van geïntegreerde kwantitatieve en kwalitatieve MRI eigenschappen in GBM. Deze

studie heeft laten zien dat het combineren van deze eigenschappen met klinische data het meest optimale prognostische model voor algehele overleving oplevert wat ook reproduceerbaar was in een extern validatie cohort. Het voorspellen van moleculaire markers met deze MRI eigenschappen leek aanvankelijk veelbelovend maar kon niet op een klinisch relevante manier gereproduceerd worden in het externe validatie cohort.

Al het bovenstaande beschrijft vooral onderzoeken die zich richten op kankercellen en de rol hiervan in tumor heterogeniteit en respons op behandeling. Ondanks dat kankercellen uiteraard een hele belangrijke rol hierin spelen is er ook steeds meer aandacht voor de tumor micro-omgeving en de rol hiervan in respons op behandeling. Met de opkomst van immuuntherapie en het grote succes in verschillende soorten kanker is dit ook onderzocht voor het GBM met tot dusver teleurstellende resultaten. Hoofdstuk 6 beschrijft de eerste resultaten van ons onderzoek waarin we een analyse op single-cell niveau hebben gedaan in een cohort van GBM patiënten. Deze studie zal de mogelijkheid bieden om de interactie tussen kankercellen en immuuncellen te bestuderen en daarbij de immuuncellen aanwezig in de tumor te kunnen typeren. Hiermee kunnen er hopelijk nieuwe strategieën ontwikkeld worden om het succes van immuuntherapie in het GBM te verbeteren.

Samenvattend, deze thesis beschrijft manieren om tumor heterogeniteit in het GBM te bestuderen. We hebben laten zien dat GBM organoïden een veelbelovend nieuw GBM model is en dat intratumorale heterogeniteit hierin behouden blijft. Het werk beschreven in deze thesis legt de basis voor toekomstig onderzoek zoals het betrekken van componenten van de tumor micro-omgeving in deze organoïden. Daarnaast hebben we de mogelijke toepassing laten zien van computer gestuurde analyses van MRI beelden zoals radiomics om prognostische en predictieve modellen te ontwikkelen. Desalniettemin is er voor beide technieken nog standaardisatie, optimalisatie en verdere validatie nodig voordat ze de vertaalslag kunnen maken naar de dagelijkse praktijk en een direct invloed kunnen hebben op de zorg voor GBM patiënten.

Addenda

Impact of this thesis

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Impact of this thesis

Despites decades of pre-clinical and clinical research, the standard-of-care treatment for patients with glioblastoma (GBM) has remained relatively unchanged and prognosis remains poor. Currently, novel approaches to study cancer biology, growth, progression and treatment effectiveness are of great relevance to identify novel treatment options and ultimately increase patients' survival. As cancer is still one of the leading causes of mortality worldwide continuous research to improve patients' outcome is of great importance. Furthermore, the societal impact of cancer including the large economic burden its treatment modalities and overall morbidity pose calls for a more effective and efficient approach towards cancer treatment.

The work in this thesis describes novel methods to study tumor heterogeneity in GBM using either imaging techniques or pre-clinical research models. Even though the studies in this thesis focus on GBM, its findings can also be extrapolated to all other types of solid cancers.

Clinical relevance

Inter- and intratumoral heterogeneity has gained a lot of attention over the past decade as a major determinant in cancer relapse and treatment resistance. In this light, the paradigm is now shifting from a one-treatment-fits-all approach towards a personalized medicine approach which takes into account genetic profiles (i.e. Mammaprint score) and specific tumor characteristics (i.e. HER2-status for targeted therapy or tumor proportion score for PDL-1 expression for immune checkpoint inhibitors). The great improvement that we have observed in cancer treatment due to these approaches shows that accounting for inter- and intratumoral heterogeneity is of great clinical relevance in order to move the field forward.

This thesis describes different methods to study tumor heterogeneity in GBM. These approaches can aid into a more accurate prediction of the most effective treatment option to provide a patients' best chances of survival. On the other hand, this also has the potential to predict whether a patient is not going to respond to a certain treatment preventing needless adverse events and subsequent loss of quality of life.

Gain for society

The findings described in this study, though focused on GBM, can be extrapolated towards other types of cancers. Being able to improve understanding about tumor heterogeneity will ultimately benefit cancer patients in general. This will also aid in more optimal treatment selection with higher chances of success regarding prolonging survival and improve quality of life. Cancer treatment and morbidity poses a large burden on the health care system and is accountable for a major part of health care costs. Especially the newer treatment options come with high costs which leads to the ethical question of how much money society is able and willing to spend for sometimes only a small benefit in survival. This has recently become a major topic for debate as also in The Netherlands we reach the limits of the health care costs society can account for. Therefore, more accurate prediction of useful treatment options leading to less morbidity and treatment costs related to ineffective treatment options are important for society as a whole, not only from a patients' point of view but also from a societal and economical view.

Improvement in health care

In line with what is said before, an improved understanding of tumor heterogeneity can both improve cancer survival as well as decrease cancer morbidity. This is relevant in health care as the morbidity that comes with the toxicity of anti-cancer treatment also poses a large burden on the already overloaded health care system.

Novelty of the concept

Both quantitative and qualitative imaging analysis and cancer organoids are not novel concepts but have been getting more attention over the past years. Even though these concepts are not new, they still hold limitations which withholds them from actual clinical implementation.

This thesis further explores these concepts and critically reviews their current limitations. Though promising, we believe that several obstacles still have to be overcome before these concepts can be implemented into clinical practice. Important overall aspects include standardization and thorough validation of the methods that are used in order to be universally applicable and to be able to make an actual impact on the way cancer is being researched, diagnosed and ultimately treated.

Road to the market

The research presented in this thesis does not directly hold market value in a commercial sense. Commercial services are already available concerning both imaging texture analysis and organoids but are currently limited to a research setting. Further development is a major topic of research across the globe. Exploring more complex organoid (and other three-dimensional) models including multiple cell types present in the tumor, and further optimization of imaging analysis, progressing into artificial intelligence and deep learning, will increase the clinical applicability of these techniques and paves the road for further commercial development.

Concluding remarks

Tumor heterogeneity is a major determinant in cancer relapse and treatment resistance, not only in GBM but in all types of cancer. The approaches described in this thesis are of relevance towards the clinical, society and the health care system. Further development of these techniques is a major research topic worldwide and will aid in progression to the market and clinical implementation.

Addenda

Acknowledgments

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Acknowledgments

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Addenda

Curriculum vitae

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Curriculum vitae

Maikel Verduin was born om April 14th 1993 in Gorinchem, the Netherlands. He obtained his secondary school degree in Nature & Health (VWO) at the Anna van Rijn College in Nieuwegein in 2010. After this he started studying Medicine at Maastricht University from which he graduated *cum laude* in 2016. As a medical doctor he started as a resident not in training at the department of Internal Medicine at the Elisabeth-Twee Steden Ziekenhuis in Tilburg.

In 2017 he started his PhD at the department of Radiotherapy and the department of Medical Oncology under supervision of prof. dr. Marc Vooijs, prof. dr. Vivianne Tjan-Heijnen and dr. Ann Hoeben. His research focusses on developing novel approaches to study tumor heterogeneity in glioblastoma. He initiated both clinical and pre-clinical studies and participated in collecting funds from several institutions. During his PhD he presented his work at multiple occasions including the National Working Group of Neuro-Oncology (LWNO) annual days in the Netherlands as well as at the Society of Neuro-Oncology (SNO) annual meeting at Phoenix, USA in 2018.

After 4 years of full-time PhD work he started as a resident in training at the department of Internal Medicine at the Elisabeth Twee Steden Ziekenhuis in Tilburg under the supervision of dr. Carolijn Klomp, dr. Marjo van Kasteren (both Elisabeth-Twee Steden Ziekenhuis) and dr. Geralt Vervoort (Radboudumc Nijmegen).

Addenda

List of publications

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List of publications

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