Biomarkers in Brain Tumors with Focus on Glioblastoma

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*You must demand of yourselves,
even if others would not demand of you!*
John Paul II, 1983

To my family

Populärvetenskaplig sammanfattning

I Sverige insjuknar varje år ca 500 patienter med gliom och de flesta drabbas av den mest aggressiva formen, glioblastom (GBM), med en median överlevnad mellan 14-17 månader, även med behandling. Män diagnosticeras upp till 60% oftare än kvinnor och i vissa studier verkar de ha sämre prognos. De flesta som insjuknar med GBM är äldre och behandlingen utgörs av en kombination av strålning och läkemedlet temozolomid (TMZ) som är ett cytostaikum. Den enda kända biologiska tumörmarkören som kan förutsäga effekt av TMZ behandling är så kallad metylering av DNA i promotorregionen till genen för ett DNA reparationsenzym, *O⁶-metylguanin DNA metyltransferas (MGMT)*. Metylering av *MGMT* medför en minskad förekomst av enzymet och en försämrad förmåga att reparera DNA skador. Det innebär att DNA skador som orsakas av TMZ inte kan repareras lika effektivt och tumörcellerna dör. Patienter med tumörer som är *MGMT* metylerade har därför bättre prognos och överlever längre. Det finns ett stort behov av att identifiera ytterligare biomarkörer som kan förklara skillnaderna mellan män och kvinnors sjukdom, men också faktorer av betydelse för effekt av strålbehandling eller för patienter med tumör som inte är *MGMT* metylerade. Dessa frågor är fokus för denna avhandling. I arbetena I-III undersökte vi könsrelaterade skillnader för GBM, kopplade bla till könskromosomerna Y och X. I arbete IV sökte vi efter så kallade metyleringsspecifika biomarkörer som kan kopplas till exceptionellt lång eller kort överlevnad hos patienter som behandlats med strålning eller TMZ.

Förlust av hela Y kromosomen (loss of chromosome Y, LOY) hos kroppscellerna har associerats till åldrande och har kunnat kopplas till ålderssjukdomar, bl a cancer. Vi undersökte 10 gener på Y kromosomen hos 105 män med GBM och som hade behandlats med TMZ samtidigt med strålning och fann att tumörcellerna ofta saknade hela eller delar av Y-kromosomen, bla kunde förlust av genen "sex determining region Y" (*SRY*) kopplas till kortare överlevnad. För att konfirmera fyndet analyserades en annan kohort av 219 GBM patienter från "The Cancer Genome Atlas" (TCGA) där lågt genuttryck av *SRY* användes som indikator på låg aktivitet och eller förlust av *SRY* genen och var starkt kopplad till kortare överlevnad.

I arbete II analyserades data från 3 olika kohorter av patient med GBM för att undersöka förekomsten av *MGMT* metylerad tumör hos män respektive kvinnor. Den ena kohorten bestod av GBM patienter från den Nordiska randomiserade fas 3 studien för patienter 60 år eller äldre, där standard behandling med 60 Gy strålning jämfördes mot strålning till 34 Gy som ges under kortare tid eller behandling med läkemedlet TMZ. Den andra kohorten var populationsbaserad och alla patienterna erhöll TMZ samtidigt med strålbehandling. Det sista patient materialet kom från TCGA där GBM patienterna erhållit olika typer av onkologisk behandling. I den populationsbaserade kohorten hade kvinnor bättre överlevnad än män och i alla 3 patient kohorterna var det fler kvinnor som hade metylerad *MGMT* gen än män.

I arbete III undersökte vi androgen receptorn (*AR*) i GBM tumörvävnad. *AR* är belägen på X-kromosomen och vi fann att genen förekom i både flera och färre kopior hos tumörcellerna, förändringar som var betydligt vanligare bland kvinnor. Genuttrycket av *AR* var generellt förhöjd i tumörvävnad utan skillnader mellan könen. Däremot hade förhöjt värde motsatt effekt beroende på kön, där kvinnor uppvisade en kortare överlevnad medan män överlevde längre tid med dessa förändringar. Metyleringsgraden hos *AR* undersöktes och vilka delar av promotorn skiljde sig mellan män och kvinnor.

I arbete IV deltog 59 patienter från den Nordiska gliom studien, jämnt fördelade utifrån behandlingsarm och metyleringsstatus på *MGMT*. Alla patienter hade goda prognostiska faktorer men kort eller lång överlevnad. Vi genomförde helgenoms metyleringsanalys och sökte efter områden i DNA med skillnader i metyleringsgrad hos patienter med kort eller lång överlevnad. Vi identifierade sådana områden i gruppen TMZ behandlade och *MGMT* metylerade, gruppen 60Gy och *MGMT* icke-metylerade samt 34Gy och *MGMT* metylerade. Inga metyleringsskillnader kunde identifieras hos patienter som behandlats med TMZ beroende på om de hade *MGMT* metylerade tumörceller eller inte. Genom att använda metyleringsprofilen hos tumörcellerna för diagnostik så kunde vi identifiera en felklassificerad tumör som inte var GBM. Slutligen kunde vi också beräkna så kallad "epigenetisk ålder" hos tumören utifrån metyleringsdata. Vi använde 3 olika algoritmer för beräkning. Vi kunde påvisa att i alla olika behandlingsgrupper så hade de med kort överlevnad en trend mot lägre epigenetisk ålder, men fynden var inte signifikanta och kräver verifiering i ett större material.

Sammanfattningsvis så utvidgar denna avhandling vår kunskap kring molekylära olikheter mellan GBM hos män och kvinnor och kopplingen mellan dessa skillnader och överlevnad hos patienterna. Våra resultat talar också för att det kan finnas ytterligare metyleringsbaserade biomarkörer av betydelse förutom *MGMT*, såsom tumörens epigenetiska ålder och som kan användas för att skilja mellan patienter med god och dålig prognos. Dom primära hjärntumörerna, gliom, är inte vanliga, men de är obotliga.

Abstract

The primary brain tumors, gliomas, are not very common but they are deadly. Each year in Sweden around 500 patients will be diagnosed with a glioma and unfortunately most of them will have the most aggressive type, glioblastoma (GBM). Median survival, even if treated, is poor (14-17 months). Males are diagnosed up to 60% more often than females and they often have a worse prognosis. GBM affects mainly older patients and treatment includes radiochemotherapy with temozolomide (TMZ). The only known predictive biomarker for TMZ treatment is methylation of the *O*⁶-methylguanine DNA methyltransferase (*MGMT*) promoter and patients with methylated *MGMT* have better outcomes. There is a great need for biomarkers to decipher existing sex differences and others that identify patients that will benefit from radiotherapy (RT) or TMZ despite of unmethylated *MGMT*.

Papers I-III are focused on investigating sex differences in GBM and in Paper IV we examined the methylation-based biomarkers used in diagnostics, on patients from the Nordic trial with exceptionally good and poor survival when treated with TMZ or RT.

Loss of the Y chromosome (LOY) in male's blood cells is associated with aging and, among other diseases, with cancer. We looked at 10 genes located on chromosome Y in 105 males with GBM treated with TMZ concomitant with RT and found that they are often deleted. Detected LOY, as well as deletion of the sex determining region Y (*SRY*) gene were associated with shorter overall survival. Low *SRY* gene expression analyzed in an additional cohort of 219 samples from The Cancer Genome Atlas (TCGA) was also associated with a shorter survival.

In Paper II we re-analyzed data from three cohorts to compare the frequency of *MGMT* methylated tumors in males and females and investigated whether sex is an important factor associated with patient's survival. This was done in a GBM cohort from the randomized, phase 3 Nordic trial, which included patients 60 years or older, treated with standard RT (60Gy) vs. hypofractionated RT vs. TMZ given in up to six 4 weekly cycles; in a population-based cohort, treated with TMZ concomitant with RT and an excerpt of the TCGA cohort of patients treated with different modalities. In all three cohorts there was a higher fraction of *MGMT* methylated tumors in females and *MGMT* methylation was predictive of longer survival for those treated with an alkylating agent, such as TMZ.

The third study investigated the androgen receptor (*AR*), located on chromosome X as a potential sex susceptibility factor for GBM. We found that the gene encoding for *AR* can be amplified or deleted in GBM, and these changes are more common in females. The *AR* gene expression was enhanced in GBM but did not differ between sexes. At the same time, in a separate analysis for males and females, we found that high *AR* expression is associated with shorter survival in females and longer survival in males. Also, the methylation sites in the *AR* promoter that correlated with gene expression are sex specific.

In Paper IV we included 59 patients from the Nordic trial, equally divided by the treatment arms and *MGMT* methylation status, with good prognostic factors and with long or short survival. We performed genome-wide methylation analysis and identified differentially methylated sites between those with long and short survival for the TMZ treated, *MGMT* methylated samples, as well as the 60Gy, *MGMT* unmethylated and 34Gy *MGMT* methylated samples. This small pilot study was unable to discern any differentially methylated sites in

TMZ treated samples with unmethylated *MGMT*, associated with long or short survival. By using a methylation-based diagnostic classifier, we were able to detect a misclassified sample that was not a GBM. Lastly, we calculated so called ‘epigenetic age’ of the tumor tissue based on the methylation data and using three different algorithms and found that in all treatment groups short-term survivors tended to have lower epigenetic age, though these results were not significant and need verification in a larger cohort.

In summary, this thesis advances our knowledge on the molecular differences between male and female GBM and the association between these alterations and patients’ survival. Our results also suggest that there are potential methylation-based biomarkers apart from the *MGMT* promoter methylation, that can be used to distinguish between patients with good and poor prognosis, for instance, the epigenetic age.

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Abbreviations

1p/19q- Short arm of chromosome 1/long arm of chromosome 19
2HG- 2-Hydroxyglutarate
AC- Astrocytoma
AKT- Protein kinase B
AMELY- Amelogenin Y-linked
AR- Androgen receptor
ARSEP1- Arylsulfatase L pseudogene 1
ASCO- American Society of Clinical Oncology
ATRX- Alpha-thalassemia/mental retardation, X-linked
BRD4- Bromodomain containing 4
CBTRUS- Central Brain Tumor Registry of the United States
CDKN2A/B- Cyclin dependent kinase inhibitor 2A/B
cfDNA- Cell-free DNA
CHEK2- Checkpoint kinase 2
CI- Confidence interval
CN- Copy number
CNS- Central Nervous System
CpG- Cytosine-phosphate-guanine
ddPCR- Droplet digital polymerase chain reaction
Dickkopf2- Dickkopf-related protein 2
DMCs- Differentially methylated CpG sites
DMGs- Differentially methylated genes
EGFR- Epidermal growth factor receptor
EGFRvIII- Epidermal growth factor receptor transcript variant III
EIF1AY- eukaryotic translation initiation factor 1A Y-linked
FFPE- Formalin fixed, paraffin embedded
FGFR- Fibroblast growth factor receptor
GABRA1- Gamma-aminobutyric acid type A receptor subunit alpha 1
GBM- Glioblastoma
G-CIMP- Glioma CpG island methylator phenotype
GLS1- Glutaminase 1
GWAS- Genome wide association study
Gy- Gray, radiation dose
HOXC4- Homeobox C4
HOXD13- Homeobox D13
HOXD8- Homeobox D8
HR- Hazard ratio
ICV- Intracranial volume
IDH1- Isocitrate dehydrogenase 1
IDH2- Isocitrate dehydrogenase 2
JAM-A- Junctional adhesion molecule A
KDM5D- lysine demethylase 5D
LGG- Low grade glioma
LOY- Loss of chromosome Y
MAPK- Mitogen-activated protein kinase

MDM1/2/4- Mouse double minute 1/2/4
 MGMT- O6-Methylguanine DNA methyltransferase
 MRI- Magnetic resonance imaging
 NEFL- Neurofilament light chain
 NF1- Neurofibromatosis type 1
 NLGN4Y- neuroligin 4 Y-linked
 NR2F2- Nuclear receptor subfamily 2 group F member 2
 ODG- Oligodendroglioma
 OR- Odds ratio
 OS- Overall survival
 p16- Cyclin-dependent kinase inhibitor 2A
 p21- Cyclin-dependent kinase inhibitor 1
 PD-1- Programmed cell death protein-1
 PD-L1- Programmed cell death protein-1 ligand
 PIK3CA- Phosphatidylinositol 3-kinase catalytic alpha polypeptide
 PTEN- Phosphatase and tensin homolog
 RB1- Retinoblastoma 1
 RT- Radiotherapy
 SLC12A5- Solute carrier family 12 member 5
 SRY- Sex determining region Y
 SVZ- Subventricular zone
 SYT1- Synaptotagmin 1
 TCGA- The Cancer Genome Atlas
 TERT- Telomerase reverse transcriptase
 TET- Ten-eleven translocation
 TFAP2A- Transcription factor AP-2 alpha
 TMSB4Y- thymosin beta 4 Y-linked
 TMZ- Temozolomide
 TP53- Tumor protein 53
 TSS- Transcription start site
 TTFs- Tumor treating fields
 USPY- ubiquitin specific peptidase 9 Y-linked
 UTY-ubiquitously transcribed tetratricopeptide repeat containing, Y-linked
 WHO- World Health Organization
 WNT- Wingless-related integration site
 ZFY- zinc finger protein Y- linked
 αKG- Alpha-ketoglutarate

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IV Łysiak M, Das J, Malmström A, Söderkvist P. Methylome analysis for prediction of long and short-term survival in glioblastoma patients from the Nordic trial. Manuscript

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Introduction

Cancer

In 2020, more than 19 million people were diagnosed with cancer and 10 million died due to the disease (1). With these numbers, cancer is one of the leading causes of death worldwide and the World Health Organization (WHO) lists efforts for new cancer treatments as essential. In the next 20 years, cancer incidence is expected to rise nearly 50%, which creates enormous challenges for the researchers to find new treatments and health care systems to accommodate the increased number of patients. Cancer is predominantly a disease of older patients, due to the long-term accumulation of risk factors. According to the most recent data from the US based National Cancer Institute's Surveillance, Epidemiology and End Results program the median age of a cancer diagnosis is 66 years (2). There is no doubt that cancer is a disease with a genetic background. The profiles of genetic alterations, e.g., point mutations, amplifications, losses of genetic material or fusions of genes, along with epigenetic modifications of the genome are cancer signatures (3, 4). These aberrations often target proto-oncogenes, which promote cell proliferation but if mutated lead to uncontrolled proliferation of cells. Genetic alterations also target tumor suppressor genes, which have inhibitory effect on the cell cycle, and when mutated, they lose the inhibitory function. However, for the full inactivation, the two-hit hypothesis predicts that both alleles of a tumor suppressor have to be affected but only one allele for a proto-oncogene. Familial syndromes, e.g., Li-Fraumeni syndrome, which are associated with increased risk of cancer, are typically caused by inherited germline mutations in tumor suppressors and a second somatic mutation in the remaining wild-type allele (5). Exposure to ionizing radiation, smoking, alcohol, poor diet, excessive exposure to UV radiation, viral infections are just some of the environmental factors that are known to increase the risk of cancer development due to accumulation of genetic changes (6). However, for many types of tumors risk factors remain unknown.

In females, the most common are cancers of the breast, colorectum, lung, cervix and thyroid, while in males lung, prostate, colorectum, stomach and liver cancers are dominating (data accessed in January 2022) (7). Overall, males are diagnosed with cancer more often than females and have worse prognosis (7, 8). Tumors of the brain and central nervous system (CNS) are on the 20th place of the most common cancers, but limited treatment options and the site of the tumor lead to one of the lowest survival rates for such patients. Gliomas are the most common primary malignant brain tumors. The reported annual incidence of gliomas in US is 6 per 100,000 and males are affected more often (9). For glioblastoma (GBM), the most common glioma, male:female ratio is 1.6:1 but little is known about the mechanisms causing this difference (9, 10).

Cancer treatment usually involves combinations of several modalities and surgery, chemotherapy and radiotherapy (RT) are the most common. Maximal safe resection is the goal when it comes to surgery for gliomas but tumor localization and the proximity to important brain structures might prevent radical removal (11, 12). For a successful treatment of any glioma, the chemotherapeutic agent needs to pass the blood-brain barrier to be able to reach the tumor. This greatly limits the number of chemotherapeutics that can be used, even if the blood-brain barrier becomes leaky during the course of the disease. RT is used alone or in combination with chemotherapy for glioma patients but while contributing to the local control of the disease, it also carries risks of adverse events (13, 14). Unfortunately, even with these

treatments, prognosis for glioma patients remains poor (13, 14). By studying genetic and epigenetic alterations, we can advance our knowledge about glioma development, including the higher prevalence in males, as well as find new treatment targets and improve identifying which patients would benefit from these treatments. Therapies targeting recurrently mutated genes have been successfully used in a variety of cancers, e.g., lung or colorectal cancer (15). Tyrosine kinase receptors, e.g., epidermal growth factor receptor (*EGFR*), are often mutated in GBM (16). Unfortunately, until now, clinical trials of tyrosine kinase inhibitors and monoclonal antibodies targeting the receptors have failed to show survival benefit for GBM patients in comparison to standard therapies (13). There is also a growing interest in immune therapies for gliomas, especially immune checkpoint inhibitors, effective, e.g., in melanoma and non-small cell lung cancer (17, 18). Inhibition of programmed cell death protein 1 (PD-1) or its ligand (PD-L1) leads to inhibition of the negative regulatory pathways of the immune system and the anti-tumor response. GBM, however, is a tumor with a low immunogenic response, and so far, no significant clinical benefit has been demonstrated for immune checkpoint inhibitors (13, 19).

The aim of the studies included in this thesis is to contribute to the development of personalized glioma patient care, focusing on biomarkers associated with sex differences, treatments and survival of GBM patients.

Biomarkers in cancer

The general meaning of a biomarker is biological characteristic that is objectively measured and evaluated as an indicator of normal biological or pathological processes, or a response to a therapeutic intervention. Hence, attempts to find biomarkers for cancer have become a priority for many researchers. Depending on the purpose, diagnostic, prognostic or predictive biomarkers can be distinguished, with the possibility of more than one category being suitable. There are countless examples of what can be used as a biomarker, starting with simple biochemical blood tests, through magnetic resonance imaging (MRI) results to transcriptome profiling of a tumor. In recent years we have seen an immense increase in the understanding of basic molecular mechanisms in tumor formation, as well as development and identification of molecular biomarkers, accelerated by the rapid development of high throughput technologies.

Gliomas

In Sweden, more than 500 patients are diagnosed yearly with glioma (20). GBM is the most common among gliomas and accounts for nearly half (48.6%) of the malignant tumors of the CNS (9). The yearly incidence rate for GBM is 3.23/100 000 and the peak is in the 6th decade of life. There is also a sex disproportion, with males being diagnosed 60% more often than females (9, 10). GBM patients have the worst prognosis, with a median survival of 14-17 months (21). As expected, comorbidities reduce survival by more than 6 months (22). Low grade gliomas (LGG) are diagnosed in younger patients, with a mean age of 41 years and a median survival of approximately 7 years. Even though prognosis is much better, patients with LGG still suffer a fatal disease (23).

Demographically, older age, being a male of European ancestry (non-Hispanic) and having higher socioeconomic status are associated with increased glioma risk (24). Gliomas are thought to be sporadic cancers, with only up to 5% having familial component (25, 26) and

only 1-2% being caused by syndromes with Mendelian inheritance, e.g. Li-Fraumeni syndrome, neurofibromatosis 1 and 2, Turcot syndrome and multiple hamartoma (24, 26). According to current reports, there are 25 loci discovered through 8 genome-wide association studies (GWAS) that can explain up to 30% of glioma incidences (24). Most of these variants can be found in the genes commonly mutated in gliomas.

Exposure to ionizing radiation is the only environmental factor known to increase the risk of gliomas (26). Respiratory allergies, asthma and eczema have been reported to reduce the risk of glioma by 30% (OR: 0.72, 95% CI: 0.58–0.90) (24, 26, 27). Also, the use of statins to treat hypercholesterolemia and use of non-steroidal anti-inflammatory drugs, in this case aspirin, are both associated with reduced risk of glioma (24). A recent meta-analysis showed no correlation between diet and risk of glioma development (28). Alcohol intake is a known risk factor for many cancers (29) and a study based on data collected from over 200 000 individuals showed a surprising reduction in glioma risk related to alcohol consumption, both, in males and in females (30). Smoking, use of cellular phones and low frequency electromagnetic fields have been suggested as risk factors, but conclusive evidence is not available (24).

Biomarkers in gliomas

Historically, gliomas are a very heterogeneous group of tumors, often causing difficulties for pathological diagnosis. Currently, they are relatively well defined at the molecular levels, thanks to the international initiatives, like The Cancer Genome Atlas (TCGA) (16, 31, 32). Although the exact etiology of gliomas remains unclear, aberrations in important cell-signaling pathways have been described (Figure 1) (16). Deregulation of these pathways leads to the acquisition of the hallmarks of cancer, which among others include uncontrolled proliferation, resisting cell death, inducing angiogenesis, evading immune destruction, deregulation of cell metabolism and capacity to metastasize, the latter being very rare in gliomas (33). For GBM, the most common chromosomal alteration is gain of chromosome 7, where the *EGFR* gene is often found amplified. In addition to amplifications, *EGFR* carries point mutations and an alternative transcript, which lacks exons 2-7 (*EGFR* variant III-*EGFRvIII*), is commonly detected. In total, more than half of GBM have alterations in *EGFR*. Highly recurrent chromosomal loss is found on chromosome 10, with common deletions and mutations of a tumor suppressor, phosphatase and tensin homolog (*PTEN*). About 10% of tumors have mutations in the neurofibromatosis 1 (*NF1*) tumor suppressor. The tumor protein 53 (p53) pathway is altered in over 80% of tumors, through deletions or mutations of *TP53*, amplifications of mouse double minute 1/2/4 homologous proteins (*MDM1/2/4*) responsible for facilitating p53 degradation and/or deletions of cyclin dependent kinase inhibitor 2A/B (*CDKN2A/B*). The *CDKN2A/B* deletions, in addition to their role in the p53 pathway, are also responsible for deregulation of the retinoblastoma 1 (Rb1) pathway, which is affected in nearly 80% of the tumors. Apart from *CDKN2A/B* deletions, the pathway is also affected by *RBI* mutations or deletions and amplification of cyclin dependent kinases 4/6 (*CDK4/6*). Aberrations in receptor tyrosine kinases, like *EGFR*, platelet derived growth factor receptor (*PDGFR*) or fibroblast growth factor receptor (*FGFR*) are complemented by mutations in the PIK3CA/Akt signaling pathway (16, 31).

These molecular alterations are reflected in the gene expression profiles, which through clustering analysis and in combination with gross genetic alterations data are used to distinguish four GBM subtypes (34). The mesenchymal subtype is enriched for *NF1*

mutations and *CDKN2A* deletions, whilst loss of chromosome 7, gain of chromosome 10 and EGFR amplifications are the main characteristics of the classical subtype. The proneural subtype collects tumors with PDGFR, TP53 alterations and includes tumors with isocitrate dehydrogenase 1 mutations (*IDH1*). The neural subtype is classified based on the expression of neuron markers, such as NEFL, GABRA1, SYT1 and SLC12A5.

Figure 1. Genes and pathways altered in GBM. Reprinted with permission from (16).

Epigenetic changes together with somatic alterations have impact on tumor development (33). Analysis of DNA methylation and clustering analysis revealed that just as transcriptomes, methylomes can be used for subclassification of GBM (16). A study from TCGA network found that by using data from DNA methylation arrays, GBM tumors can be divided into 6 subtypes, partially overlapping with the subtypes defined by the transcriptomes and correlating with the somatic alterations. This gave the foundation to another classification of CNS tumors, published by Capper et al., who used genome-wide methylation profiling to create a methylation-based CNS tumor classifier and a further subclassification of GBM, IDH wild-type tumors into 8 subclasses (35). Despite high expectations for a universal brain tumor classifier, several studies which used a single-cell or spatial biopsy analysis approach have shown that gliomas still remain heterogeneous when it comes to transcriptome and methylome profiles (36-39).

Comprehensive studies of LGG found that mutations in the *IDH1/2* genes are very common in these tumors, as well as mutations in *TP53*, alpha-thalassemia/mental retardation, X-linked (*ATRX*) and co-deletions of the 1p/19q chromosomal arms (32). Both, *IDH1/2* mutations and 1p/19q co-deletion are considered as biomarkers for good prognosis.

Results of these studies finally led to an update of the previous CNS tumor classification, enforced since 2007 (40). The updated classification, which for the first time included molecular biomarkers next to the histological review of the tumor tissue, was introduced by

WHO in 2016 and has been recently amended (14, 41). The current algorithm for classification of diffuse astrocytic and oligodendroglial glioma is depicted in Figure 2. The first-line molecular assessment includes *IDH1/2* mutations, which separates astrocytomas (AC) and oligodendrogliomas (ODG), both IDH-mutated, from IDH wild-type GBM tumors (from here on referred to as GBM).

Figure 2. Current diagnostic algorithm for diffuse gliomas with amendments to the classification introduced in 2016. Reprinted with permission from (14). MVP- microvascular proliferation, H3.3G34- histone H3.3 glycine 34, H3K27M- methionine substitution of lysine at residue 27 of histone H3, MGMT- O6-methylguanine–DNA methyltransferase, TERT- telomerase reverse transcriptase, WHO- World Health Organization

The *IDH1/2* genes are coding for an enzyme, which participates in the tricyclic acid cycle. IDH1 is located in the cytosol and peroxisomes, while IDH2 is found in the mitochondrial matrix. They are responsible for catalyzing the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG). In gliomas, missense mutations most commonly affect codon 132 of *IDH1* and codon 172 of *IDH2*, and these mutations are mutually exclusive. These two variants alter the IDH enzymatic activity resulting in the formation of D-2-hydroxyglutarate (2HG) instead of α -KG. Accumulation of 2HG inhibits the activity of multiple dioxygenases, histone demethylases, and the ten-eleven translocation (TET) family of 5-methylcytosine hydroxylases. TET enzymes participate in the DNA demethylation process and inhibition leads to accumulation of methylation of CpG sites throughout the genome (42). This, together

with inhibition of histone demethylases, introduces epigenetic changes that are specific to IDH-mutated tumors. In fact, IDH mutations are associated with a glioma cytosine-phosphate-guanine (CpG) island methylator phenotype (G-CIMP) and these tumors have a better prognosis (43).

MGMT methylation

DNA methylation in gliomas is important for one more reason and it mainly concerns GBM tumors. For patients treated with an alkylating agent temozolomide (TMZ), methylation in the promoter region of the *O*⁶-methylguanine–DNA methyltransferase (*MGMT*) gene is associated with an improved overall survival (OS) and is used as a predictive biomarker (13, 14, 44, 45). *MGMT* participates in DNA repair by removing alkyl groups from the O⁶ position of guanine. Methylation of the promoter region regulates gene transcription and availability of the protein. The increased methylation leads to decreased levels of the protein this resulting in a less effective DNA repair, reinforcing the cytotoxic and apoptotic effect of the treatment with an alkylating agent (44). Current guidelines for treatment of gliomas highlight the importance of *MGMT* promoter methylation as a biomarker for informed treatment selection (13, 14).

TERT mutations

Mutations in the promoter of telomerase reverse transcriptase (*TERT*) are the most common mutations found in GBM, in up to 86% (46, 47). Two mutually exclusive hotspots are located -124bp and -146bp upstream of the transcription start site (TSS) and both are C>T transitions, which create DNA consensus sites enabling binding of transcription factors and increased expression of the gene (46, 48). *TERT* promoter mutations as well as *TP53*, *EGFR*, and *PTEN* mutations are believed to occur early during gliomagenesis because they were found in matched GBM tissue samples and the distant subventricular zone (SVZ), which is believed to be the place of origin of gliomas (49). *TERT* promoter mutations have been associated with shorter OS (46), although results presented at the European Association of Neuro-Oncology Meeting 2021 revealed no correlation between *TERT* mutations and clinical outcomes (OS, progression free survival) (47), questioning the prognostic nature of this biomarker. Due to their prevalence in GBM, mutations in *TERT* promoter can be used as a tumor marker in cell-free DNA (cfDNA) in liquid biopsies and a detection method with a sensitivity of over 60% has been recently described (50). *TERT* mutations, next to *EGFR* and *IDH* mutations are markers for sporadic gliomas, as in tumors caused by exposure to ionizing radiation, these mutations do not occur (51).

Treatment

Standard of care

After positive diagnostic MRI, for all gliomas, maximal safe resection is preferred (11, 12, 14). If this approach is not possible due to the risks it carries, a biopsy should be performed instead, with very few exceptions (14). This will allow for a diagnosis and choice of the therapy.

For GBM, concomitant radiochemotherapy with temozolomide is considered a standard treatment regimen (52). It consists of TMZ administered daily (75 mg/m²) for 6 weeks together with RT in fractions of 2Gy per day weekdays for 6 weeks to a total of 60Gy. This is followed by up to 6 cycles of TMZ given in 150-200 mg/m² days 1-5 in each cycle (52). The

only predictive biomarker for TMZ treatment is methylation of the MGMT promoter (44, 45) but TMZ is used also for patients with unmethylated MGMT in this setting (Figure 3) (14).

Figure 3. Algorithm for treatment selection for GBM IDH wild-type patients. In each case, an appropriate clinical trial can be considered instead of the treatment recommended by the algorithm. Addition of tumor treating fields for patients who have received radiotherapy concurrent with temozolomide can also be considered (53). Reprinted with permission from (14). KPS- Karnofsky performance status

Deviations from concomitant radiochemotherapy with temozolomide are dictated by the presence of unmethylated *MGMT* and poor performance status. Hypofractionated RT is a treatment offered especially to elderly patients with unmethylated *MGMT*, who are unfit for combination therapy. Cases with methylated *MGMT* are offered TMZ only. For patients over 65-70 years with a good performance status hypofractionated radiochemotherapy with TMZ can be considered. There are currently no predictive biomarkers for RT (14).

Current recommendations encourage enrollment of patients into clinical trials to accelerate finding of new treatments (14). This entails biomarker analysis upon enrollment (as entry requirements), which is especially important for targeted treatments, as well as post-hoc subgroup analyzes of biomarkers.

Treatment at progression/recurrence is dictated by the treatment, which the patient has received for the newly diagnosed tumor, performance status and neurological function. The use of humanized vascular endothelial growth factor antibody, bevacizumab, has been shown

to extend progression free survival and help to decrease peritumoral edema but does not influence the overall survival, therefore it is not approved in all countries for treatment of the recurrent GBM (13).

Tumor treating fields

At first, tumor treating fields (TTFs) were received quite skeptically by the neuro-oncology community, but this modality has since been proven to significantly extend life of GBM patients. It is a non-invasive treatment modality that acts through low-intensity (1–3 V/cm), intermediate-frequency (100–300 kHz), alternating electric fields which are delivered via cutaneous transducer arrays worn by the patient for the majority of the day. The alternating electric fields exert force on dipole molecules, which interferes with, e.g., microtubule polymerization during mitosis leading to reduced proliferation (54). Sweden is one of a few countries offering TTFs to patients. Importantly, TTFs were shown to extend survival of GBM patients by 5 months when added to concomitant radiochemotherapy with TMZ in the primary setting (53, 54).

Sex differences in gliomas

Male dominance in cancer is present throughout many malignancies (8, 55). Among primary CNS tumors, all gliomas are more frequently diagnosed in males than females, whereas meningiomas are more common in females (9). GBM is diagnosed 60% more often in males (10). Some studies have suggested survival advantage of females, but recent reports do not support these claims (10, 56, 57).

Figure 4. Factors underlying sex differences in gliomas, which are discussed in detail in the section Sex differences in gliomas.

Molecular differences

Many genetic variants are associated with glioma risk, and several studies report on sex-specific variants as well. A GWAS from 2018 showed that rs11979158 carries a risk only for males and rs55705857 is more strongly associated in females (58). A study published recently

revealed that female-specific association can be found for some *TERT* variants and male-specific associations are found for certain *EGFR* variants (59).

There is a larger sex disparity in mesenchymal and neural subtypes of GBM than in classical and proneural (60). A mesenchymal GBM model (NF1-/dominant negative p53 mice) derived from males is reported to exhibit, after EGF treatment, a higher stem-like behavior and proliferation rate in comparison to the model based on female cells. The study disclosed that these effects were mediated by the sex differences in the inactivating phosphorylation of the Rb protein, which was greater in male mice (60). Another study found that the sex differences of Rb regulation are dependent on the p21 and p16 activity in astrocytes (61). Both, p21 and p16 are cyclin dependent kinase inhibitors (62). Once cyclin dependent kinase inhibitors are activated, they prevent Rb phosphorylation and inhibit cell cycle progression (62). In a GBM mouse model, in response to DNA damage, female astrocytes had a higher activity of p21 and p16 leading to cell cycle arrest, whereas male astrocytes continued to proliferate (61). The *TP53* gene, named the guardian of our genome, is mutated in more than half of GBM (34). Haupt et al. showed that the p53 pathway is associated with male dominance in various types of cancer, which included a higher frequency of *TP53* mutations, higher frequency of mutations of p53 negative regulators located on the X chromosome, both in males, and lower expression of the X chromosome mutated alleles in females (63). The importance of p53 has also been emphasized in a recent study by Rockwell et al., who reported that 6 codons of *TP53* carry mutations at a sex-specific frequency; 4 codons more frequently mutated in females and 2 in males (64). This further led to the activation of different transcription programs depending on the sex of the mouse astrocytes (64).

Another described example of a difference with a molecular background is the association between high expression of Frizzled-7, a Wnt receptor, and poor survival of only male GBM patients, despite the presence of high Frizzled-7 expression in female tumors as well (65).

Zhang et al. reported that female gliomas (GBM and LGG) have higher mutation burden than male tumors, which can partially be attributed to the high mutation frequency of the X chromosome (66). This TCGA-based study also showed that the mitogen-activated protein kinase (MAPK) pathway is characterized by clonal mutations in female GBM, and that receptor tyrosine kinases are more likely to have clonal mutations in male LGG (66). Another TCGA-based analysis revealed transcriptomic sex differences, with integrin signaling being the most specific for females and cell cycle regulation pathway being male specific (67).

In addition to the intrinsic molecular differences between the sexes in GBM, there are also differences that occur in the tumor microenvironment. Microglia, one of the components of the tumor microenvironment, interacts with tumor cells through the junctional adhesion molecule A (JAM-A), which helps to maintain the GBM cancer stem cells (68). Interestingly, this may be true only in the male tumors, as JAM-A deficiency in female mice injected with GBM cells induced pro-tumorigenic microglia behavior (69).

Epigenetics

The embryonic development of the brain, as any other tissue, requires epigenetic changes (70, 71). DNA methylation is the most widely studied component of epigenetics. Importantly, the acquired epigenetic marks can differ between sexes (72) and be dictated by sex hormones, e.g. it has been shown that treatment with testosterone-propionate of mouse embryonic neural

stem cells leads to a global decrease of DNA methylation in both, XX and XY cells (73). In the developing mammalian brain, researchers found a decreased activity of DNA methyltransferases in males compared to females, contributing to the sex differences in methylation (74). As neural stem cells of the SVZ are thought to be involved in the gliomagenesis (49), one should expect epigenetic sex differences in the tumors as well. In fact, a recent study based on TCGA data showed that methylation of CpG sites differs between sexes in all glioma tumors (75). In all female gliomas, hypermethylated differentially methylated CpG sites (DMCs) were found in the CpG island regions, associated with gene promoters. In contrast, in males, the hypermethylated DMCs were primarily localized in the so-called open sea regions. The same study proposed that in male GBM, hypermethylation affects proapoptotic genes, leading to their down-regulation and in females, hypermethylation provides protective effect by down-regulating cell migration genes (75).

A few studies suggested that *MGMT* promoter methylation, described above, was associated with longer survival, but only in females, and that the fraction of patients with methylated *MGMT* is significantly higher among female patients (65, 76). We explored this subject further in Paper II of this thesis.

Silencing of one of the X chromosomes is possible thanks to the long non-coding RNA *Xist* (77). About 15% of the genes escape the inactivation (78) and recent reports show that a portion of the escaping genes can act as tumor suppressors or oncogenes (79, 80), thus likely contributing to sex disparity in cancer. One of the studies showed that a skewed inactivation of the X chromosome measured in the location of the androgen receptor (*AR*), leads to increased risk for developing high-grade glioma in young females (<40 years) (81).

The bromodomain containing 4 (*BRD4*) gene codes for a protein that binds to the acetylated histones and aids accumulation of enhancers to drive transcriptions of genes (82). As an epigenetic regulator, Brd4 was found to bind different enhancers in male and female astrocytes of the GBM mouse model; oncogenes and stem cell markers in males and tumor suppressors in females (82). This translated into increased proliferation in Brd4 knock-down in females and decreased proliferation in males.

Sex hormones

It can be argued that the hormonal influence on glioma development is subtle, if any. There is no sudden increase in the incidence of gliomas during and after puberty (83) and data on the increased risk of glioma due to exposure to exogenous hormones are inconclusive, although they tend to lean towards protective properties of estrogens (84). However, *in vitro* and *in vivo* studies indicate that by affecting signaling pathways of sex hormones, we may be able to modulate response to radio- and chemotherapy (85, 86). Zhou et al. showed that GBM cells overexpressing estrogen receptor β were sensitized to TMZ treatment (85), and Werner et al. reported that inhibition of the AR sensitizes GBM cells to radiotherapy (86). With the current knowledge, sex hormones are not considered as a causative factor behind the sex disparity in gliomas, but they do not seem to be neutral to the disease either.

Metabolism

One of the hallmarks of cancer cells is increased glucose metabolism via anaerobic glycolysis, despite conditions appropriate for oxidative phosphorylation (87). A difference in metabolic activity of males and females can be seen already early in life, during embryogenesis (88).

Ippolito et al. hypothesized that metabolic sex differences may even exist in glioma (89). In a study, based largely on TCGA collection of LGG, they found that increased expression of a set of 11 genes involved in the glycolytic pathway, defined as high-glycolytic group, correlated with short OS. However, this was only observed in males, as no such correlation was found in females, even though the levels of transcripts were similar in both sexes.

In a very recent report, the same group analyzed the glutamine metabolism and showed that glutamine uptake is higher in male GBM (90). Additionally, the glutaminase 1 (GLS1) enzyme, which metabolizes glutamine to glutamate, was upregulated in male specimens and in the *in vitro* experiments only male cells exhibited sensitivity to GLS1 inhibition. Using a dataset of 757 metabolites analyzed in 76 GBM patients, Sponagel, et al. also noted a general male bias towards amino acid and carbohydrate metabolism and a female bias towards lipid metabolism (90), which is consistent with observations made in adult healthy individuals (91). This suggests that future treatment approaches targeting tumor metabolism should also consider the sex of the patients, e.g., antiglycolytic agents or GLS1 inhibitors could be more suitable for male patients.

Response to treatment

One of the adverse effects related to TMZ treatment is myelosuppression and females are at higher risk of severe myelotoxicity (92), which might be caused by the differences in drug metabolism. This is also consistent with the general observations about adverse effects of cancer treatments in females (93). However, females seem to respond better to the standard of care treatment regimen (surgery, focal RT and TMZ), as shown by Yang et al. (67). Significant reduction in GBM growth rate was found there only in females, following TMZ treatment, despite an equal sex distribution of initial tumor growth velocities. This having a direct impact on OS differences. Unfortunately, this study did not account for the effect of *MGMT* promoter methylation status in the analysis.

The use of immunotherapy as cancer treatment offers many new possibilities, but its use in GBM is still limited due to unsatisfying efficacy (94). It has been suggested that sex differences in the immune system may translate into different efficacy of immunotherapy (95), e.g., studies in other cancers suggest that immune checkpoint inhibitors could bring more benefits to males (95, 96), but it is yet to be properly evaluated for GBM patients.

Anatomical differences

A Norwegian group hypothesized that higher incidence of glioma is associated with a larger size of the brain, which is the effect of the higher number of stem cell divisions (97). On average, male brains are larger than female brains and in this study brain size was expressed as the intracranial volume (ICV) assessed through the MRI scans. Not only glioma patients had larger ICV than the control group, but also, male patients had larger ICV than females. The study also reported that for any person, an increase in the ICV by 100ml resulted in the increase of odds ratio for a high-grade glioma diagnosis by 1.7-fold (95% CI: 1.44–1.98, $P < 0.001$) (97). Height is thought to correlate with the brain size and a study conducted in Israel showed that increased adolescent height is a risk factor for glioma development in males but not for females (98). Billelo et al. reported that GBM is located more frequently in the right temporal lobe in females and the left side is more common in males (99). Also, female tumors were significantly larger than male tumors (15.02cm^3 vs. 11.701cm^3 , $p=0.005$). Another

study found a sex difference in the frontal and temporal location of GBM, with males more likely to have tumors in the frontal lobe and females more likely to be affected in the temporal lobe (100). Additionally, combined analysis for both sexes showed that tumors in the frontal lobe correlated with better overall survival in comparison to those located in the temporal lobe.

Socioeconomical differences

This thesis is based on three papers (I-III) that deal with the molecular aspects of sex differences in GBM, but the socio-economic determinants related to the patient's sex cannot be overlooked, as they may affect diagnosis, treatment and survival. Socioeconomic differences are present even in countries with universal healthcare systems (101). In Sweden, there is a unique opportunity to study diseases in such a broad spectrum thanks to the comprehensive registers. Data published in 2016 from a Swedish population-based registry of Stockholm-Gotland region for primary intracranial tumors showed that male patients had more inpatient days (overnight stays at the hospital) one year after diagnosis (27.2 days; CI 95%: 24.4–29.9) in comparison to females (21.8 days; CI 95%: 19.7–23.9) (22). For the outpatient visits (any consultancy without overnight stay) one year after diagnosis, the difference was even more striking, with 51.6 days (CI 95%: 47.8–55.3) for male patients and 35.6 days (CI 95%: 33.1–38.2) for female patients, even though there was no difference between sexes for the in- and outpatient days one year before the diagnosis. It should be noted, however, that the analysis did not separate the sexes in high- and low-grade tumors, and males are more often diagnosed with high grade gliomas. Interestingly, the same study showed no differences between sexes in the lead time from diagnosis (understood here as a first radiologic suspicion of the malignancy) to surgery, surgery to histopathological report and surgery to start of non-surgical treatment (chemo- and radiotherapy). A later study based on the Swedish registry, which included only LGG, also showed no sex differences regarding waiting time, surgical procedure or number of surgery-related complications that would lead to re-operation, despite worse preoperative performance status of females and higher fraction of asymptomatic male patients (102). Different models of healthcare systems undeniably influence access to treatments. In a recent study based on the Central Brain Tumor Registry of the United States (CBTRUS), analysis of more than 170 000 patients revealed that males more often received chemotherapy, radiotherapy and surgery but still had a higher risk of death (103). Gliomas, especially GBM, have poor prognosis and palliative care is often the only option. An American study found that female patients are more often admitted to hospice to improve end-of-life care than males and that males stay in hospice for a much shorter time when admitted (104).

Biomarkers of survival

Apart from sex, the most important clinical prognostic factors for gliomas include patient's age, type of surgery and performance status (12, 20, 45). Type of diagnosis is also of importance, and it is closely related to the age at diagnosis, as GBMs are often diagnosed in older patients, whilst AC and ODG are more common in younger patients. Maximal safe resection is preferred in all gliomas over a biopsy, regardless of molecular subtype (12). Genetic alterations, currently used to define the diagnostic entity, namely *IDH1/2* mutations and 1p/19q codeletion, were shown to have predictive value for gliomas, especially for patients treated with a combination of procarbazine, lomustine and vincristine (105, 106). For

LGG, *IDH1/2* mutations and 1p/19q codeletion also had prognostic value, and this observation led to the updated brain tumor classification, valid today (32, 41).

The greatest struggle to extend the survival of patients concerns GBM. Unfortunately, a recent meta-analysis did not show any improvement in the 5-year survival rate for GBM over the past 15 years, when focusing on patients who survived at least 2 years (107). However, the same study showed increasing number of patients reaching 2- and 3-year survival over the studied period (107). Having cohorts with too few long-term survivors is the main issue for successful analysis of the factors that predispose to the exceptionally long survival. Despite the obstacles, a few reports are available, suggesting the importance of clinical and molecular biomarkers. A great hope is put in the ETERNITY study (EORTC 1419), which collects data about patients with survival over 5 years from centers in Europe, US and Australia. The first results presented at the American Society of Clinical Oncology (ASCO) meeting in 2019 showed that MGMT promoter methylation and gross total resection were favorable factors and median age of included patients was 52 years, which is lower than average for a population-based cohorts (108). However, we will have to wait for a full analysis of molecular and clinical factors.

One of the studies investigating factors associated with long survival revealed that survival of 3 years or longer is predominantly associated with younger age (<50 years) and MGMT promoter methylation, although tumors with staining of Ki-67 below 20%, a nuclear protein and marker of proliferation, also seemed to have a better chance for longer survival (OR: 3.07; 95% CI: 1.15-8.29; $p=0.026$) (109). Another study, which compared patients with survival below 1 year and longer than 3 years, showed that age (<60 years, $p=0.001$), total resection ($p<0.001$) and tumor localization (no contact with SVZ, $p=0.05$) were predictors of long survival (110). With age being such a strong prognostic factor, some tried to answer, whether there are cellular programs that differ between younger and older patients. Bozdag et al. suggested that tumors of older patients have a signature of genes involved in angiogenesis and response to hypoxia and that age-based differences can be found on the genetic, transcriptomic, and epigenetic level (111). A recent study showed that the most common type of mutation in GBM is a C>T substitution, closely related to aging, and that mutational profiles of gliomas largely depend on the patient's age, e.g. there is association between *EGFR*-gains and loss of *CDKN2A* in GBM and mutations in *ATRX* in LGG (4). The inability of cells to repair damaged DNA is believed to be a major cause of aging (112), which further supports the strong position of age as a prognostic factor. For GBM tumors, single nucleotide variant count increases at an average of 0.018 mutations per megabase per year (4).

The use of epigenetic signatures to select patients with a better prognosis was proposed by several research groups (43, 113, 114). With the current knowledge, a striking majority of G-CIMP tumors harbors *IDH1/2* mutations. Before the inclusion of *IDH1/2* mutations into the glioma classification scheme, high-grade gliomas with *IDH* mutations were often treated and analyzed together with the current diagnostic group of GBM. Analysis of methylation profiles of long- and short-term survivors from such a mixed cohort identified a G-CIMP profile as a prognostic biomarker of long survival, which was additionally associated with IDH (115). Single CpG loci in homeobox gene family (*HOXD8*, *HOXD13* and *HOXC4*), transcription factors (*NR2F2* and *TFAP2A*) and a Wnt pathway negative regulator (*Dickkopf2*) were also proposed in this study as a mean to distinguish long- and short-term survivors. More groups

explored the use of DNA methylation analysis to select GBM patients with better prognosis, but the suggested biomarkers seemed to be true only for the specific study (113, 114).

Seizures are a common and unpleasant symptom accompanying brain tumors, but surprisingly, seizure presentation at diagnosis was found to be associated with longer survival of GBM patients, regardless of treatment for seizure management (116). However, these results should be considered cautiously, due to inclusion of a small group of patients with *IDH* mutations.

Aims

The generalized aim of the studies included in this thesis is to find biomarkers associated with sex differences, treatments and survival of glioma patients, which could contribute to further development of personalized glioma patient care.

The specific aims for this thesis are:

- To identify molecular biomarkers on sex chromosomes associated with sex differences in GBM (Paper I & III)
- To investigate the role of *MGMT* promoter methylation in GBM sex disparity (Paper II)
- To identify prognostic and/or predictive methylation-based biomarkers for patients treated with TMZ or RT (Paper IV)

Comments on Materials and Methods

A detailed description of materials and methods used in Paper I-IV can be found in the appropriate section of each paper. Only brief comments on the most relevant methods can be found here.

Patients and samples

Patient material used in all studies included in this thesis was collected upon signed informed consent and in accordance with the ethical permissions as stated in Papers I-IV. All studies were conducted retrospectively, and each study included data from TCGA as an additional/confirmatory cohort, which is especially important when analyzing biomarkers in limited sample sized cohorts. Samples used in Paper I and III were collected consecutively since 2008 at Linköping University Hospital but few samples from the period 2004-2008 were also included. Patients included in Paper IV were selected from a randomized trial and cohorts included in Paper II were from the randomized trial and a consecutive collection at Linköping University Hospital. All tumors were routinely characterized for *IDH1/2* and *TERT* mutations with Sanger sequencing, in addition to the diagnostic analysis performed by the pathologists.

Droplet Digital Polymerase Chain Reaction (ddPCR)

The idea behind droplet digital PCR (ddPCR), which is the use of limiting dilution for quantitative PCR amplification detection, was first published in 1992 (117). The microfluidic system for ddPCR analysis, currently used worldwide and used in the analyzes described in Paper I and III, was commercialized by BioRad in 2011, with subsequent updates and automation of sample preparation.

In principle, ddPCR is a step-up from the standard quantitative real-time polymerase chain reaction (q-PCR), which uses a fluorescent signal to measure amplification of DNA. This also defines the array of possible applications for ddPCR, e.g., analysis of gene copy number (CN), relative and absolute gene expression, detection of rare mutations, quantification of libraries for next generation sequencing and quantification of viral load (118-120). Amplification of the target is detected either with fluorescently labelled TaqMan probes or with a fluorescent dye EvaGreen. Many suppliers offer optimized, ready-to-use primers and probes, however, it is also possible to design them in-house using freely available online tools. Each sample is partitioned into 20,000 droplets (water-oil emulsion). Reagents necessary for target amplification are in the aqueous phase of the droplets, together with the fragmented and randomly dispersed DNA template. After end-point PCR, each droplet is passed through a reader to determine whether amplification occurred (positive droplets) or not (negative droplets), by measuring the fluorescence signal. Finally, the fraction of positive droplets is fit into Poisson distribution to return the concentration of target DNA in the analyzed sample.

ddPCR was used in Paper I and III to analyze copy number (CN) alterations and relative mRNA gene expression. Each experiment was based on a comparison of the amplification of a target to a reference, whether it was a gene with 2 stable copies for CN analysis or stable mRNA expression for gene expression analysis. In Paper I, we used probes targeting 10 genes spread throughout chromosome Y and analyzed their CN alterations. If deletion was detected in all of them, we assumed a loss of chromosome Y (LOY). Previously, we used a similar approach to detect 1p/19q co-deletion in glioma samples (121).

Formalin fixed, paraffin embedded (FFPE) samples are often used for biomarkers studies, as it is the easiest way to archive them. Unfortunately, such sample preparation leads to poor quality and fragmentation of DNA. The ddPCR method is suitable for analysis of DNA from FFPE samples, as amplicons are rarely longer than 100 base pairs. Additionally, thanks to the high sensitivity and low input requirements, ddPCR is now often considered for liquid biopsy analyzes (50, 122).

Methylation arrays

Methylation of mammalian DNA occurs at cytosines followed by guanines, called CpG sites. Methylated CpG sites are in general a sign of inactive chromatin and accumulation of CpG sites is often found at the transcription regulatory regions, that is gene promoters.

As mentioned before, DNA methylation is an important part of biomarker research in brain tumors- methylation of the *MGMT* gene promoter is used as a predictive biomarker for treatment with TMZ (123) and genome-wide methylation profiles can be used to aid tumor classification (35).

The proof of concept for the genome-wide methylation analysis using microarray technology has been published in the early 2000s (124). Over the years, not only has the number of CpG sites included in the microarray design increased, but there has also been an incredible development of bioinformatics tools for data analysis.

In Paper IV, we used the Infinium MethylationEPIC BeadChip arrays, which allow for quantitative interrogation of over 850,000 methylation sites in each sample. The protocol (Figure 5) starts with bisulfite conversion of DNA extracted from cells or tissue (snap frozen and FFPE material is suitable). During this process methylated cytosines remain intact and unmethylated cytosines are deaminated to uracil. In a subsequent genome-wide amplification, during the synthesis of a new strand, methylated cytosines are paired with guanine and uracil with thymine, resulting in changes to the original DNA sequence. Next, DNA is fragmented, purified through isopropanol precipitation, and then resuspended in a buffer. In this form samples are loaded onto arrays and DNA hybridizes to the complementary oligonucleotides (probes) attached to the surface of immobilized beads. Each bead is coated with oligonucleotides specific to one locus. In the next step, probes are extended by a single, chain terminating dideoxynucleotide, based on the complementarity to the bound DNA fragment. Incorporated dideoxynucleotides are labelled with biotin (guanine and cytosine) or dinitrophenyl (DNP) (adenine and thymine) and labels will allow to distinguish between genotypes. Next, DNA fragments are removed, and the staining process begins. Green, fluorescent streptavidin binds to biotin and the red fluorescent anti-DNP antibody binds to DNP. Additional rounds of staining allow for amplification of fluorescent signal. After the staining process, arrays are imaged on the scanner and data is collected in the raw intensity files, also called .idat files. These files are compatible with the methylation-based classifier available online (35) and can be used for further analysis, e.g., differential methylation analysis, as described in detail in Paper IV.

Figure 5. A flow-chart representing processing of the samples during Illumina's methylation array analysis. This method is suitable for snap frozen and FFPE samples, but the latter require small modifications of the protocol. After DNA isolation, DNA degradation from FFPE samples is assessed through a qPCR run and compared to a control DNA. The additional restoration step for FFPE samples provides larger fragments for the following whole genome amplification.

Maximally selected rank statistics

When using continuous data as a biomarker, such as gene expression, an appropriate cut-off point must be selected for clinical decisions to be made. For this purpose, the most common is to use arbitrary dichotomization, dividing the data by the median, quartiles, or the mean value of the expression (125). Even in the case of the already extensively studied effect of *MGMT* promoter methylation on survival of GBM patients (44, 45), there is still no clear consensus on the value of the cut-off point for the methylated and unmethylated tumors (126). The method of maximally selected rank statistics allows for dichotomization of quantitative, ordinal and censored data (127, 128). First, we assume a simple relationship between the X quantitative variable and the Y response variable, where the value of the quantitative variable determines creation of the two groups in the response variable (one with X values below or equal to the cut-off point and one with X values above the cut-off point). In Papers I and III, the method was used to find the best cut-off points for the quantitative factors (mRNA gene expression, CN data) that separated the response variable (OS of the patients). For each possible value of the X , the standardized log-rank statistics is calculated, and the highest value of the standardized log-rank test indicates the best cut-off point (Figure 6). It is important to define reasonable sizes of the two groups, e.g., not to allow for one group to be smaller than 10-20% of the studied population.

Figure 6. An example of the application of maximally selected rank statistics from Paper I. The normalized mRNA expression values of the SRY gene obtained from microarray data were used as the X quantitative data and the OS served as the Y response data. For each value of X , the standardized log-rank statistic was calculated using the maxstat package in R 3.6.0, RStudio version 1.3, and the highest value of standardized log-rank statistic corresponded to the cut-off point of *SRY* mRNA expression 3.3867(log2). The obtained dichotomization revealed survival differences between patients with low and high SRY expression (11.9 vs. 14.7 months, respectively).

Results and discussion

Paper I

The existing male dominance observed in GBM patients is a complex outcome of many factors. In Paper I, we report on our findings from a retrospective, cross-sectional study about loss of chromosome Y (LOY) in male GBM, CN changes of the 10 selected genes from the Y chromosome (Figure 7a) and the influence of both on OS.

Patient material for CN analysis with ddPCR, blood and FFPE tumor tissue, was collected in the South-East Sweden hospital region between 2008-2016. These patients, referred to as a Swedish cohort, were from a previously published study and all had confirmed GBM, IDH wild-type tumors (129). All were treated with TMZ concomitant with RT. Tumor samples for 105 patients and 61 matched blood samples were available. The second cohort was derived from TCGA collection and consisted of transcriptome data of 219 male primary GBM, IDH wild-type, accessed via the GlioVis portal (130, 131). Correlation between changes in the copy number of genes in the Swedish cohort, the expression level of these genes in the TCGA cohort and OS was tested with Kaplan-Meier log-rank analysis after choosing the best cut-off value with maximally selected rank statistics.

The CN analysis of the 105 GBMs revealed that chromosome Y was subjected to various gene dosage alterations. We found deletions and amplifications of the tested genes, but unidirectional changes of all markers were found only in 6 samples for deletion and 4 for amplification (Figure 7b). Our study revealed that deletion of the sex determining region Y (*SRY*) gene significantly correlated with shorter OS (10.8 vs. 14.8 months, $p=0.0031$; $n=104$) (Figure 7c) and in the multivariate analysis, which included age, preoperative performance status, *MGMT* promoter methylation status and type of surgery, *SRY* deletion had the strongest effect on survival ($HR=3.352$; 95% CI: 1.695–6.629; $p=0.001$). Further, we showed that these changes were almost exclusively present in the tumor tissue and not in the peripheral blood and were not age dependent. Deletions of all markers, considered as a LOY, also correlated with significantly shorter OS of the patients (7 vs. 14.5 months, $p=0.0016$; $n=94$) (Figure 7d).

From the analysis performed on TCGA cohort, which tested the correlation between mRNA expression of the 10 markers on chromosome Y and patient survival, we were able to confirm the link between *SRY* and reduced OS. Median OS for patients with low *SRY* expression was 11.9 months and for those with high expression 14.7 months ($p=0.00069$). The significantly shorter OS was also associated with low expression of *TMSB4Y* (12.6 vs. 15.8 months, $p=0.0023$), *AMELY* (12.7 vs. 14.2 months, $p=0.0087$) and *UTY* (12.7 vs. 18.5 months, $p=0.0031$). Interestingly, through the gene set enrichment analysis we showed that low expression of *SRY* was associated with several signaling pathways involved in GBM development, e.g., EGFR, PDGFR and Myc (34).

There were no data available on the prevalence of LOY or deletions of specific genes of chromosome Y among GBM patients, hence the cross-sectional design seemed suitable. In the first study reporting on LOY in the peripheral blood of the elderly men from the Uppsala Longitudinal Study of Adult Men, LOY was found in 8.2% of the cohort (132). Another study on head and neck cancer revealed LOY in 28.6% of tumors (133). This influenced our lack of

prior power calculations, which are otherwise highly desired in biomarker studies exploring the relationship of the biomarker with survival.

Figure 7. Deletion of markers on chromosome Y and LOY are associated with shorter survival of male GBM. Representation of the markers tested with ddPCR on chromosome Y (a). All 10 markers were successfully analyzed in 94 samples; deletions were detected with the cut-off CN<0.8 (red) and amplifications with the cut-off CN>1.2 (blue) (b). Maximally selected rank statistics was used to investigate the relationship between the CN of genes and OS, and Kaplan-Meier analysis in (c) shows that deletion of *SRY* (CN<0.6) was associated with shorter OS (10.8 vs. 14.8 months). LOY was defined as deletion of all 10 markers and was detected in 6 samples. These patients also had shorter OS (7 vs. 14.5 months) (d). *SRY*-sex determining region Y, *ZFY*- zinc finger protein Y- linked, *AMELY*- amorgenin Y-linked, *ARSEP1*- arylsulfatase L pseudogene 1, *USPY*- ubiquitin specific peptidase 9 Y-linked, *UTY*-ubiquitously transcribed tetratricopeptide repeat containing, Y-linked, *TMSB4Y*- thymosin beta 4 Y-linked, *NLGN4Y*- neuroligin 4 Y-linked, *KDM5D*- lysine demethylase 5D, *EIF1AY*- eukaryotic translation initiation factor 1A Y-linked.

It may also be viewed as a disadvantage to analyze deletions on the DNA level in one cohort and mRNA expression as the approximation of the loss in the other. Ideally, the confirmatory

cohort should be treated in the same way as the discovery cohort, with the exact same analyses performed. However, deletions commonly lead to reduced mRNA expression of the gene, hence, we looked for a similar effect of both on OS. Also, eight out of ten markers used here had a good correlation between gene expression and CN in a study based on head and neck squamous cell carcinoma (133). The approach of using markers spread throughout the chromosome was previously used by Mitchell, et al. (134) but a disadvantage of our methodology was the use of one reference probe, as it is often advised to use two or more. Extreme downregulation of chromosome Y expression has been shown before as a risk for glioma development and was associated with *EGFR* amplifications and *EGFR* overexpression (135). Wright, et al., showed that LOY is associated with 19 autosomal genetic regions, which also associate with loss of chromosome X in females (136). In a different study, a group of genetic variants associated with LOY occurrence was identified in the proximity of common cancer related genes, like *CHEK2* or *TERT* (137). The same genetic variants led to increased risk of glioma (OR 2.36; 95% CI: 1.34-4.17; $p = 0.004$) and when tested in women, they were associated with increased risk of breast, ovarian and endometrial cancer. These results support the presence of a generalized genetic instability related to the occurrence of LOY. Although LOY and SRY deletions are not common events in GBM, both strongly correlated with decreased OS, suggesting their prognostic value for male GBM patients.

Paper II

Methylation status of the *MGMT* promoter is a known predictor of successful TMZ treatment (44, 45) but has not been systematically investigated for possible sex differences. Few studies pointed towards a higher prevalence of *MGMT* promoter methylation in females, which could be the reason for the better results observed among females (56, 76). In Paper II we report on the re-analysis of two published GBM cohorts and a TCGA-derived cohort, for which we assessed sex disparity of *MGMT* promoter methylation, OS, and the associations between them.

The first cohort consisted of 342 patients from the Nordic trial, for whom *MGMT* status was available for 203 (45). The randomized Nordic trial compared three treatment arms, TMZ given in up to six 4 weekly cycles (200mg/m², day 1-5 every 28th day) vs. standard RT 60Gy administered over six weeks vs. hypofractionated RT of 10 fractions of 3.4Gy to the final dose of 34Gy. In the second cohort, there were 179 GBM, IDH wild-type patients, all treated with postoperative RT concomitant with TMZ, and *MGMT* promoter methylation status was available for all (129). Lastly, in TCGA collection, we identified 257 GBM, IDH wild-type patients with known *MGMT* status and 189 of them were treated with an alkylating agent (TMZ or nitrosourea), which is of importance for the *MGMT* methylation status and survival assessments.

Despite a lower number of females in all three cohorts, we found a higher proportion of methylated *MGMT* tumors among them (48%-59% in females vs. 33%-36% in males, $p < 0.05$). In the Nordic trial cohort, *MGMT* promoter methylation status was predictive of survival only in the TMZ arm and we noted only a borderline survival benefit for females (Figure 8a). In the multivariate analysis performed separately for males and females from the TMZ arm, which included *MGMT* status, preoperative performance status, age and type of surgery, we found that tumor resection was associated with longer survival for females

($p=0.017$), and methylated MGMT ($p=0.013$) with preoperative performance status ($p<0.0001$) were associated with longer survival in males.

Figure 8. Survival curves for females and males with different *MGMT* promoter methylation status. Survival analysis was done with the Kaplan-Meier log rank method for the TMZ arm (n=72) of the Nordic trial and longest survival was found for females (9.7 months, CI7.5- 11.9) and males (9.0 months, CI7.4–10.6) with methylated MGMT, compared to females (7.5 months, CI2.9–12.0) and males (6.8 months (CI5.8–7.9) with

unmethylated MGMT but differences did not reach statistical significance ($p=0.056$) (a). In the population-based cohort treated with radiochemotherapy females with methylated MGMT had the longest survival of all groups and the differences were statistically significant ($p = 0.0002$) (b). Similarly, we found a significant survival difference between males and females treated with an alkylating agent with mMGMT in the TCGA cohort ($n=189$, $p=0.004$) (c). uMGMT- unmethylated MGMT promoter, mMGMT- methylated MGMT promoter.

In the population-based cohort, a female survival benefit was noted already in the analysis stratifying patients only by sex, which was further strengthened by the separation according to the MGMT status (Figure 8b). Multivariate analysis again showed a strong association between tumor resection and survival for females (radical surgery vs. biopsy/partial resection HR: 0.33, 95% CI: 0.13-0.84; $p=0.02$), whereas methylated MGMT correlated with longer OS in both sexes (males: HR=0.61, 95% CI: 0.4-0.94, $p=0.024$; females: HR=0.39, 95%CI: 0.22-0.70, $p=0.0014$).

Results from the TCGA cohort revealed longer survival of females with methylated MGMT in comparison to the rest of the cohort ($n=257$, females with methylated MGMT vs. females with unmethylated MGMT vs. males with methylated MGMT vs. males with unmethylated MGMT; 17.2 months vs. 11.8 months vs. 12.7 months vs. 12.6 months, respectively, $p = 0.01$). This was also true in the analysis which only included patients treated with alkylating agents ($n=189$) (Figure 8c).

The results of this study confirmed a higher proportion of MGMT methylated tumors in females. In the population-based cohorts we found that females had a survival benefit even if MGMT status was not included, a result not observed in the Nordic trial. Recent data from CBTRUS and a Swedish National Quality Registry for Primary Brain Tumors also do not support the overall survival benefit in females (10, 57). It should be pointed out though, that the Nordic trial only included patients 60 years or older, whilst the other two cohorts consisted of younger patients as well. A recent US based study showed that in all age groups (0-80+) there is a higher incidence of GBM in males but the survival advantage for females disappears for patients 70 years or older (83). We did not observe an age influence on OS in the multivariate analysis performed in the Nordic cohort, but the analyzed groups were small. At the same time, in our population-based cohort, patients were divided by the median age (58 years for males and 59 years for females), making it difficult to capture the effect. However, the correlation between sex and age should be considered for future studies.

Another limitation that should be considered is that *MGMT* methylation was measured with a different method in each cohort, which may cause discrepancies, especially with the lack of consensus for the cut-off distinguishing methylated tumors from the unmethylated ones (126).

In summary, this study showed that females have *MGMT* promoter methylated tumors more frequently than males and MGMT tumors are associated with better survival. We also demonstrated the importance of considering a subgroup analysis to elucidate potential sex differences in glioma patients. Such analyzes are increasingly required and the data from already published studies can be a valuable source for investigation.

Paper III

Sex chromosomes are commonly understudied due to the abundance of repetitive sequences and problems with handling compensation for gene dosage differences. The AR coding gene is located on chromosome X and before this study was initiated, several published papers have already shown overexpression of AR in GBM and the promising effects of AR inhibition (86, 138-140). However, molecular analyses were limited to CN and mRNA/protein expression analyzes, without extensive exploration of sex differences. Therefore, in Paper III, we focused on many of the AR-oriented molecular analyzes in GBM and the correlation between their results and OS in a sex-specific way.

We used samples collected consecutively at Linköping University Hospital, as well as data from TCGA database. The local sample collection consisted of 179 FFPE GBM from a previously described cohort (129), with DNA available for 170 of them. These samples were used for CN analysis. For further CN analysis, as well as gene expression and AR promoter methylation analysis, we used 91 fresh-frozen samples. We also had access to 167 blood samples from GBM patients that were mostly matched with FFPE and fresh-frozen tumor samples. Patients overlapped between these groups and in 23 cases, samples from the same patient were found in all three cohorts. Blood samples were used to analyze the number of trinucleotide repeats (CAG) in exon 1 of AR.

From TCGA, we included 354 samples from primary, IDH wild-type GBM tumors with transcriptomic data available.

By using ddPCR, we established changes in the CN of *AR* in FFPE and fresh-frozen samples and found that GBM tumors harbor deletions and amplifications of the *AR* gene, which are more common in females. In males, CN correlated with *AR* mRNA expression, but the same relationship was not found in females. Nonetheless, *AR* mRNA expression was enhanced in GBM samples of both sexes, with no differences between males and females in either analyzed cohort. In the TCGA cohort the highest *AR* expression was found in the classical subtype, according to the classification by Wang et al. (141) (Figure 9a). The CN alterations of the *AR* gene were previously reported by Zalcman et al., although they were more frequent than in our analysis, which most likely is caused by the methodological differences (138). Previous studies also found similar expression levels of AR in males and females (138, 142), but the more surprising result was increased testosterone levels in the serum of GBM patients (140).

Methylation of the promoter regions is responsible for regulation of gene expression. Here, methylation of 17 CpGs upstream of the TSS of the *AR* gene was analyzed with pyrosequencing combined with the gene expression results. Spearman correlation analysis revealed that methylation of different CpGs is linked with *AR* expression in males and females. There were three CpGs found in females, where methylation negatively correlated with mRNA expression (chrX:67543502, chrX:67543517 and chrX:67543659). In males, significant negative correlation was found for chrX:67543299 and chrX:67543895.

We used TCGA gene expression data to check whether *AR* expression has influence on the survival of males and females. For the potential cut-off selection, we used maximally selected rank statistics (as in Paper I), followed by the log-rank Kaplan-Meier analysis. This revealed that high *AR* expression in females was associated with shorter survival (high vs. low *AR* mRNA expression, 13.6 vs.15.7 months, $p=0.035$) (Figure 9b). Surprisingly, the association between *AR* expression and OS was the opposite in males and high *AR* expression correlated with better outcome (high vs. low *AR* mRNA expression, 16.6 vs.12.2 months, $p=0.04$) (Figure 9c). This was an important finding, as in the analysis combining both sexes, there was no correlation between gene expression and survival.

Figure 9. AR mRNA expression in GBM samples from TCGA cohort divided by subtypes and patients sex (a) and Kaplan-Meier curves for females (b) and males (c) with low and high AR mRNA expression divided by cut-off values indicated by maximally selected rank statistics. * $p<0.05$; ** $p<0.005$

AR regulates the expression of many genes, and to reveal the differences between males and females, we decided to go for the gene set enrichment analysis, to try to find pathways associated with high AR expression that may be involved in creating the sex differences among GBM patients. The only significant result was the enrichment of DNA repair genes in male high AR-expressing samples. The same set of genes, although present in the analysis of the female samples and $p<0.05$, had a high false discovery rate $>50\%$. These findings were strengthened by the positive correlation between AR protein expression and expression of proteins involved in the DNA repair mechanism (p53, Rad50, Chk1). In prostate and breast cancer, AR signaling mediates resistance to RT and use of antiandrogens improves the

outcome (143, 144). A study by Werner et al., showed that use of antiandrogens in AR-positive GBM cell lines and mouse xenograft models helped to overcome radioresistance, which has important clinical implications (86).

The coding region of exon 1 of the *AR* gene contains a highly polymorphic stretch of polyglutamine-coding CAG repeats (145). The number of repeats ranges from 6 to 39 in healthy individuals. AR binds to DNA via the zinc-finger domain and interacts with co-activators or co-suppressors of transcription. The length of the polymorphic region influences the transactivation capacity of AR and interaction with the mentioned co-factors (145). As prostate cancer is largely dependent on AR signalling, a study of CAG repeats showed increased risk of prostate cancer with lower numbers of CAG repeats (146). This prompted us to the analysis of CAG repeats in the DNA extracted from the blood of GBM patients. We used capillary electrophoresis and fragment length analysis of the amplified polymorphic region and compared results obtained from GBM patients (n=167) with the results from a cohort of healthy individuals (n=799), but no differences could be found. This led to the conclusion that there is no association between the number of CAG repeats and risk of GBM.

The main take home message from this study was that *AR* mRNA expression is associated with survival of GBM patients in a sex dependent manner, despite no differences in the expression between sexes. The CN alterations of the *AR* gene are common in GBM, but they do not influence the patient's OS.

Paper IV

The only known predictive biomarker for TMZ treatment in GBM patients is *MGMT* promoter methylation and no biomarkers are available for predicting response to RT (44, 123). Elderly patients, unfit for radiochemotherapy, can be treated with TMZ or RT alone, depending on the *MGMT* promoter methylation status (13, 14). However, the outcome of treatment varies even for patients with the same prognostic factors and the knowledge here is scarce.

Material from the Nordic trial, which compared three treatment arms (TMZ vs. hypofractionated RT 34Gy vs RT 60Gy), was used in Paper IV to try to find methylation-based biomarkers that could help identify patients with expected long or short survival for the same treatment modality and with the same prognostic factors. In the study in total, 59 patients were included, 20 from the TMZ and 34Gy arms respectively and 19 patients from the arm treated with RT 60Gy. All patients had good performance status (WHO 0-1) and had undergone tumor resection. Patients were also chosen to represent the longest and the shortest survival from each treatment arm. Half from each subgroup had methylated *MGMT*. Tumor DNA was subjected to the analysis with Infinium MethylationEPIC BeadChip array for methylation profiling.

First, we checked which classes and subclasses were present in our samples using a methylation-based classifier (35). In 3 cases, the content of tumor cells was too low, and samples were classified as normal tissue or tumor microenvironment, which highlights the importance of using high-purity tissue for molecular analysis. The remaining samples, apart from one, were classified as GBM, IDH wild-type and 21 of them were assigned to one methylation subclass. In 34 samples more than one subclass was detected and this stands in line with studies reporting on spatial heterogeneity of GBM samples (36, 39). One sample

was classified as pilocytic astrocytoma, which is a grade I tumor, without IDH mutations and with a very good prognosis (9). The mentioned sample was selected due to the long survival of the patient. Our finding shows the importance of integration of methylation-based classification into future clinical practice and clinical trials.

After removing the non-GBM samples, we examined the differentially methylated CpGs within each treatment arm and MGMT methylation group, but no differences were found. Then, we decided to remove samples with the cause of death related to complications (such as infections), as these most likely did not reflect the biology of the tumor itself. Finally, we selected samples of the 3 most-extreme values for long-term and 3 short-term survivors, respectively, from each treatment arm and MGMT methylation group and compared their methylation profiles for differentially methylated CpGs (DMCs). DMCs were found in the TMZ arm, with methylated MGMT; 34Gy, with methylated MGMT; 60Gy, with unmethylated MGMT and in the combined RT arm (34Gy+60Gy), with unmethylated MGMT. The structural annotation showed a similar distribution of DMCs throughout the genome in all groups (Figure 10) and we deemed that analysis important because cancers are known to display aberrant methylation patterns which are sequence and location specific (3). For instance, a recent report showed accumulation of hypermethylated CpGs in the CpG island regions of GBM and in LGG such accumulation was observed in the more distanced regions to CpG islands, namely CpG shores (3).

Due to the high numbers of DMCs and despite the very few samples, we followed with further filtration and looked only at the regions in the proximity to gene promoters (the 5'-untranslated region (5'-UTR) together with TSS200 and TSS1500) and with methylation β -values distanced by ± 2 standard deviations from the mean value calculated for each analytical group. Filtered DMCs were transformed into differentially methylated genes (DMGs) and used in the pathway enrichment analysis. Consistent with previous reports was the finding of the hypermethylated Wnt signaling pathway in the long-term survivors, in the MGMT methylated tumors from the TMZ arm (115). The Wnt signaling pathway has been shown to be of importance in GBM due to its contribution to maintenance of GBM cancer stem cells and the role played in treatment resistance (147).

Chai et al. proposed a 31-gene methylation signature for patients with unmethylated MGMT that would benefit from TMZ (148). Unfortunately, we did not identify any DMCs for the MGMT unmethylated tumors from the TMZ arm with long-term survival. Sole RT is recommended for elderly patients unfit for combination treatment and with MGMT unmethylated GBM (13). Our analysis of the combined RT arms did not yield any DMCs which could define patients with long or short survival. However, a recent study based on TCGA and the Chinese Glioma Genome Atlas cohort of 50 patients treated only with RT suggested a predictive methylation signature (149).

Figure 10. Distribution of structurally annotated DMCs found in different treatment groups. TSS200- 200 bases upstream transcription start site, TSS1500- 1500 bases upstream transcription start site, UTR-untranslated region, IGR- intergenic region

Aging of the tissue is reflected by the acquired epigenetic changes (150). As it has been pointed out several times throughout this thesis, GBM is tightly connected with age and aging-associated mutations (4), hence, we also found it appropriate to analyze the so called epigenetic age of the tumor samples. Three different algorithms were used for this purpose (150-152) and a common discovery for all of them was a trend towards lower epigenetic age of the short-term survivors in comparison to the long-term survivors from the same treatment arm and with the same MGMT status, although the differences were not statistically significant (Figure 11). Two studies, which also used a signature of 353 CpG loci of Horvath's epigenetic clock showed that epigenetic age is associated with the molecular subtypes of gliomas and patients' survival (153, 154). Interestingly, one of the mentioned studies found epigenetic age acceleration of GBM samples (epigenetic age > chronological age) (154), which was in line with our results, and the other reported epigenetic age deceleration in GBM (epigenetic age < chronological age) (153). The PhenoAge and Hanmmum's clocks(151, 152) used alongside Horvath's clocks in our study, also showed a common deceleration of epigenetic age. These discrepancies can be explained by differences in the models estimating epigenetic age and the heterogeneity of samples from published studies.

Figure 11. Epigenetic age calculated for long- and short-term survivors with different algorithms (Horvath (a), Hannum (b), PhenoAge (c)). Acceleration on the y-axis defines the difference between the epigenetic age and chronological age. LTS- long-term survivors, STS- short-term survivors, m-MGMT- methylated MGMT promoter, u-MGMT- unmethylated MGMT promoter

The results of this study require further evaluation and confirmation in a larger, well-defined cohort, because unambiguous conclusions should not be drawn from such a small number of

samples. Despite this, they can still be considered valuable, as both, hypermethylation of the Wnt pathway in long-term survivors and epigenetic age deceleration in short-term survivors point towards the importance of stem cell programs in GBM. Not only gliomas have been shown to arise from the SVZ, where neural progenitor cells can be found (49), but also, the proximity of the tumor to SVZ was associated with patients' survival (110). Moreover, single-cell analysis of GBM revealed existence of cellular states linked to neurodevelopmental cell types and these cellular states were powered by the dysregulated epigenetic mechanisms (39, 155).

Concluding remarks and future perspectives

Sophisticated, high-throughput technologies that have been developed in recent years and allow for rapid data acquisition and biomarker analysis. They also forced a shift from the analysis of a single biomarker to formulation of complex models with a great potential. The success of these changes, however, should be considered through the prism of changes that have been permanently introduced into clinical practice or are just one step away. Over a decade of intensive research, mostly focusing on single biomarkers, has led to an improvement in the brain tumor classification system, introduced in 2016 into the clinic (41). Thanks to technological development, just two years later, we were presented with an opportunity to improve this system through adding methylation profiling (156). Although the methylation classifier is not officially recommended as a diagnostic tool, it is already widely used to aid diagnosis in clinical trials or for problematic samples. A great example was presented in Paper IV, where one sample previously diagnosed as GBM, was in fact, with a methylation-based classification diagnosed as a pilocytic astrocytoma, a tumor with much better prognosis than GBM. To create a reliable classification system, there is a need for a tremendous number of samples and worldwide collaboration but with the freely available, ever-evolving classifier from the Heidelberg group, this may not be an obstacle. Methylation analysis can also be used for epigenetic age calculation and as presented in Paper IV and supported by other studies, epigenetic age could potentially serve as a quantitative prognostic biomarker for gliomas (153).

Accumulating evidence supports the use of certain biomarkers due to their prognostic and/or predictive value and newly registered clinical trials often use biomarkers as inclusion criteria, either focusing on patient groups with very limited choices or patients suitable for targeted therapies. We also observe a surge in the clinical trials that explore targeted therapies. One such phase 1 study explored the use of an inhibitor of mutant IDH1, ivosidenib, and showed a decrease in tumor size in 67% of 35 patients and a favorable influence on progression free survival in non-enhancing gliomas (157). Another phase 1 trial, NOA16, looked at a vaccine targeting IDH1 mutation (R132H) in 32 patients with grade 3 and 4 astrocytomas and found response in 93% of the patients, as well as a good safety profile of the treatment (158). Recently, genome-wide methylation profiles and other molecular biomarkers were found to be useful prognostic biomarkers in identifying patients with poor prognosis in the CATNON trial, which included non-codeleted 1p/19q anaplastic gliomas treated with RT $\pm$ concurrent and/or adjuvant TMZ (159).

As especially GBMs are fast growing tumors, the aim is to keep lead times as short as possible to reduce the time between radiologic diagnosis and start of oncological treatment. Methylation analysis could facilitate the diagnostic procedure by both determining the definitive diagnosis and provide information of biomarkers of importance to selection of therapy, such as *MGMT* promoter methylation status. From my perspective, the real bottleneck for the use of the methylation classifier in clinical practice is the access to and cost of the analysis. The use of Illumina EPIC array, together with a robust methylation classifier, can deliver a final diagnosis within a week from surgery. Luckily, already today, we see alternatives that can reduce this time to below 24 hours, namely nanopore sequencing (160, 161). A nanopore sequencer uses a flow cell with hundreds of nanopores embedded in a dielectric membrane. Changes in the electrical current are recorded whilst single DNA or RNA strands, as well as their modifications are detected. This allows for a wide range of

possible applications: DNA sequencing (gene panels, up to entire genome), methylation detection and RNA sequencing (162). Nanopore sequencing is also suitable for analysis of gene fusions which are understudied in gliomas but are an appealing treatment target (163, 164).

Important research into liquid biopsies for gliomas is also underway. Several studies have shown successful use of peripheral blood as liquid biopsy for early detection and profiling of distant cancer (e.g., GBM, pancreatic cancer, non-small cell lung cancer, colorectal and breast cancer) (165). For patients with brain tumors cerebrospinal fluid and/or blood contain tumor associated cells, cell free DNA and RNA, and other tumor derived biosources, such as extracellular vesicles (EV) and tumor educated platelets (TEPs). Their analysis will provide a less invasive and hopefully powerful source for early detection, monitoring of disease progression and treatment efficiency in a near future (122, 165, 166).

Through Papers I-III, we wanted to increase our knowledge about the molecular causes for sex disparity in GBM patients and we looked for potential sex-specific prognostic biomarkers. In Paper I we showed that deletion of the *SRY* gene and LOY, both detected in the tumor tissue, are associated with shorter survival of males with GBM. The survival advantage of females with GBM was revealed in Paper II, although reported in only one cohort. However, there was a higher frequency of MGMT methylated tumors in females. Paper II and III showed that many sex-specific characteristics of gliomas may go undetected, if sex is not considered as an important factor and included in the analyzes. Both papers showed that re-analysis of established cohorts and clinical trials can remedy this research gap. The main findings from Paper III are the more frequent *AR* CN changes in female GBM, increased expression of *AR* in GBM of both sexes and surprising sex-specific effect of *AR* gene expression on survival. This also suggests that GBM might have a larger hormone-dependent component, even if to date published reports show inconclusive results.

With increased knowledge of the role of sex differences in glioma, in the future sex specific treatment options might be developed, that will improve survival for both men and women with malignant brain tumors.

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Appendix (Paper I-IV)

Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

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