KATRI MÄKELÄ

Expression of Tumor Microenvironment Related Molecules C4d, Hsp27, Polysialic Acid and NCAM in Astrocytic Gliomas

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ABSTRACT

Background: In malignant progression a tumor microenvironment is formed in the extracellular matrix. Amongst others, inflammation and hypoxia are present. Cell connections are loosened and cells are released to migration. Complement is one pathway for activation of the immune system, which adversely modifies the tumor microenvironment. C4d is a marker for the site of complement activation. It is a degradation product of activated complement factor C4. Hypoxia induces the expression of the hypoxia-inducible factor 1-alpha (HIF-1alpha) that is a transcription factor for heat shock protein 27 (Hsp27) and carbonic anhydrase IX (CA IX). Hsp27 and CA IX enable cancer cells to survive and proceed in malignancy. Polysialic acid (polySia) adheres to the neural cell adhesion molecule (NCAM), which has been suggested to loosen cell to cell and cell to extracellular matrix connections. An isocitrate dehydrogenase 1 (*IDH1*) mutation has been recently found in astrocytomas, yet it is unclear how it affects the expression of these molecules. Diffusely infiltrating astrocytomas are highly malignant brain tumors and the patient prognosis is poor.

Aims: Immunohistochemical expression of C4d, Hsp27, polySia and NCAM was studied in astrocytic tumors in order to describe the expression patterns and to study their relationship to clinicopathological features. Their relationship to CA IX and HIF-1alpha expression, as well as *IDH1* mutation was also tested. Altogether the aim was to gain knowledge on the molecular profile of astrocytomas. The molecular profiling could also provide guidance to tumor typing and grading as well as in terms of development of astrocytoma targeting therapy.

Results: In diffusely infiltrating astrocytomas, C4d expression was found to correlate with increasing malignancy, patient survival and tumor recurrence. The C4d expression was significantly more extensive in pilocytic astrocytomas than in diffusely infiltrating astrocytomas. Expression of Hsp27 and HIF-1alpha as well as the *IDH1* mutation status correlated significantly with the tumor grade. Hsp27 and HIF-1alpha were

associated with increasing malignancy, whereas the *IDH1* mutation was associated with a lower tumor grade. The *IDH1* mutation correlated significantly with HIF1-alpha expression and CA IX expression, and negatively with Hsp27 expression. Furthermore, HIF-1alpha expression correlated significantly with Hsp27 and CA IX expression. When only *IDH1* mutated tumors were studied, HIF-1alpha correlated with CA IX expression, however in the case of *IDH1* non-mutated tumors this correlation was not found. Hsp27, CA IX and HIF-1alpha were associated with a worsened patient outcome, whereas *IDH1* mutation indicated a better outcome than if the tumor was *IDH1* non-mutated. Expression of polySia was rare in pilocytic astrocytomas. Patients with polySia expressing astrocytomas had better prognosis than patients with polySia negative astrocytomas. PolySia expression was also found to associate with *IDH1* mutation; tumors having *IDH1* mutation were more often polySia positive than negative. Additionally, NCAM positive tumors were more often *IDH1* mutated than non-mutated.

Conclusion: Expression of C4d in diffusely infiltrating astrocytomas shows how inflammation adversely affects the patient prognosis and how it is associated with increasing malignancy. In future C4d expression in tumor sample could be advisory to favor immunosuppressive or antitumor immune response enhancing therapy. Expression of Hsp27 may serve as a guideline to assessing the tumor grade and to predicting patient prognosis. Expression pathways of Hsp27, CA IX, polySia and NCAM seem to vary according to the *IDH1* mutation status. Hsp27, HIF-1alpha and CA IX could be potential target molecules in the future therapy of diffusely infiltrating astrocytomas, since they all seem to be associated with shorter patient survival.

Keywords: Astrocytoma, complement, C4d, isocitrate dehydrogenase 1, heat shock protein 27, carbonic anhydrase IX, hypoxia-inducible factor 1-alpha, polysialic acid, neural cell adhesion molecule, expression, prognosis, tumor microenvironment, hypoxia, inflammation, epithelial-mesenchymal transition

TIIVISTELMÄ

Tausta: Syövän edetessä syöpäsolujen ympärille kehittyy kasvaimelle ominainen mikroympäristö, jossa muun muassa hypoksia ja tulehdus ovat tärkeinä tekijöinä. Mikroympäristön toiminta aiheuttaa kasvainsolujen irrottautumista ympäristöstään, johtaen migraatioon. Komplementtikaskadi aktivoi tulehdussolukkoa, joka puolestaan muokkaa kasvaimen mikroympäristöä edistäen kasvaimen pahanlaatuistumista. C4d on komplementtikaskadin C4-osatekijän hajoamisessa vapautuva molekyyli. Sen esiintymistä voidaan pitää merkkinä komplementin aktivoitumisesta. Hypoksia aiheuttaa kasvainsoluissa hypoksian indusoima tekijä-1 alfan (HIF-1alfa) ekspression, joka toimii transkriptiotekijänä lämpöshokkiproteiini 27:lle (Hsp27) ja hiilihappoanhydraasi IX:lle (CA IX). Hsp27:n ja CA IX:n toiminta edesauttaa kasvainsolukon kykyä pysyä elossa ja pahanlaatuistua. Polysialohappo (polySia) kiinnittyy neuraalisen solun adheesiomolekyylin (NCAM) ulkoiseen osaan. Tämä väljentää solujen kiinnittymistä alustaansa. On epäselvää vaikuttaako kasvaimen isositraattidehydrogenaasi 1:tä (*IDH1*) koodaavan geenin mutaatio edellä esiteltyjen molekyylien ilmentymiseen. Diffuusisti infiltroivat astrosytoomat ovat pahanlaatuisia aivokasvaimia, joiden täydellinen poisto kirurgisesti on käytännössä mahdotonta epätarkkarajaisen kasvutavan vuoksi ja potilaiden ennuste on huono.

Tavoitteet: Tavoitteena oli tutkia C4d:n, Hsp27:n, polySia:n ja NCAM:in esiintyvyyttä astrosytoomissa sekä niiden esiintyvyyden yhteyttä kliinis-patologisiin muuttujiin. Haluttiin myös selvittää, vaikuttaako *IDH1*-mutaatio edellä mainittujen molekyylien ilmentymiseen ja niiden yhteyttä CA IX:n ja HIF-1alfan ilmentymiseen. Laajemmalti ajateltuna tavoitteena oli selvittää astrosytoomille ominaisia molekylaarisia poikkeavuuksia. Kartoitettuja molekylaarisia ominaisuuksia voitaisiin tulevaisuudessa käyttää apuna kasvaindiagnostiikassa ja uusia hoitomuotoja suunniteltaessa.

Tulokset: Diffuusisti infiltroivissa astrosytoomissa C4d:n esiintyvyys korreloi tilastollisesti merkitsevästi kasvaimen pahanlaatuisuuden, potilaan eloonjäämisennusteen ja kasvaimen uusiutumisen kanssa. C4d:n esiintyvyys oli voimakkaampaa hyvänlaatuisissa pilosyyttisissä astrosytoomissa kuin pahanlaatuisissa diffuusisti infiltroivissa astrosytoomissa. Hsp27:n ja HIF-1alfan ilmentyminen sekä *IDH1*-mutaatio korreloivat kasvaimen graduksen kanssa. Hsp27 ja HIF-1alfa ilmentyivät laajemmin pahalaatuisissa kasvaimissa, kun taas *IDH1*-mutaatio liittyi kasvaimen matalampaan gradukseen. *IDH1*-mutaatio liittyi merkitsevästi HIF-1alfan sekä CA IX:n esiintyvyyteen ja negatiivisesti Hsp27 esiintyvyyteen. Myös HIF-1alfa korreloi Hsp27:n ja CA IX:n esiintyvyyden kanssa. Korrelaatio HIF-1alfan ja CA IX:n välillä säilyi, kun *IDH1*-mutatoituneet kasvaimet tutkittiin erikseen, mutta se ei ollut enää merkitsevä tutkittaessa vain *IDH1*-mutatoitumattomat kasvaimet. Hsp27:n, CA IX:n ja HIF-1alfan esiintyvyys liittyi tilastollisesti merkitsevästi potilaiden lyhempään eloonjäämisaikaan. *IDH1*-mutaatio kasvaimessa ennusti pidempää elossaoloaikaa kuin *IDH1*-mutatoitumattomuus. PolySia:n esiintyvyys oli harvinaista pilosyyttisissä astrosytoomissa. PolySian ja NCAM:in esiintyvyydet ennustivat diffuusisti infiltroivaa astrosytoomaa sairastavien potilaiden pidempää eloonjäämisaikaa. PolySia:n esiintyvyys oli tavallisempaa *IDH1*-mutatoituneissa kasvaimissa kuin mutatoitumattomissa. Myös NCAM:ia ilmentävät kasvaimet olivat useammin *IDH1*-mutatoituneita kuin mutatoitumattomia.

Johtopäätökset: C4d:n ilmentyminen diffuusisti infiltroivissa astrosytoomissa osoittaa tulehduksen aiheuttamien muutosten vaikutuksen kasvaimen epäedulliseen kehitykseen. C4d:n esiintyvyys kasvaimessa voi puoltaa anti-inflammatorisen tai kasvaimen vastaista immuunipuolustusta vahvistavan hoitomuodon valintaa. Tulevaisuudessa Hsp27:n esiintyvyyttä voidaan käyttää hyödyksi kasvaindiagnostiikassa ja potilaan eloonjäämisennusteessa. Hsp27:n, CA IX:n, polySia:n ja NCAM:in esiintyvyys poikkesi *IDH1*-mutatoituneiden ja *IDH1*-mutatoitumattomien kasvainten välillä. Hsp27, HIF-1alfa ja CA IX ovat potentiaalisia syöpähoidon kohdemolekyylejä, sillä ne kaikki vaikuttavat potilaan elinajanennustetta lyhentävästi.

Avainsanat: astrosytooma, komplementti, C4d, isositraattidehydrogenaasi 1, lämpösokkiproteiini 27,

hiilihappoanhydraasi IX, hypoksian indusoima tekijä 1 alfa, polysialohappo, neuraalisen solun adheesiomolekyyli, ekspressio, ennuste, kasvaimen mikroympäristö, hypoksia, tulehdus, epiteeli-mesenkyymi-transitio

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numbers.

I Mäkelä K, Helén P, Haapasalo H, Paavonen T (2012); Complement activation in astrocytomas: deposition of C4d and patient outcome. BMC Cancer. 12:565.

II Mäkelä KS, Haapasalo JA, Ilvesaro JM, Parkkila S, Paavonen T, Haapasalo HK (2014); Hsp27 and its expression pattern in diffusely infiltrating astrocytomas. Histol Histopathol. 29:1161-8.

III Mäkelä K, Nordfors K, Finne J, Jokilammi A, Paavonen T, Haapasalo H, Korja M, Haapasalo J (2014); Polysialic acid is associated with better prognosis and IDH1-mutation in diffusely infiltrating astrocytomas. BMC Cancer. 14:623.

ABBREVIATIONS

alpha-KG	Alpha-ketoglutarate
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
CA IX	Carbonic anhydrase IX
CC	Chemokine subfamily
CI	Confidence interval
CIMP	CpG island methylator phenotype
CNS	Central nervous system
CRPs	Complement regulatory proteins
CXC	Chemokine subfamily
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Endothelial growth factor receptor
EMT	Epithelial-mesenchymal transition
GBM	Glioblastoma multiforme
FIH	Factor inhibiting HIF
Hsp27	Heat shock protein 27
HIF-1alpha	Hypoxia-inducible factor 1-alpha
IDH1	Isocitrate dehydrogenase 1
IHC	Immunohistochemistry
IL	Interleukin
Ki-67/MIB	Cellular marker for proliferation
LOH	Loss of heterozygosity
MAC	Membrane attack complex
MDSC	Myeloid-derived suppressor cells
NADP	Nicotinamide adenine dinucleotide phosphate
NCAM	Neural cell adhesion molecule
PDGFR	Platelet driven growth factor receptor
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1

PHD	Propyl-4-hydroxylases
PolySia	Polysialic acid
PTEN	Phosphatase and tensin homolog
TAM/Ms	Tumor associated macrophages/microglia
TGF-beta	Transforming growth factor beta
TMA	Tissue microarray
TP53	Tumor protein 53
Treg	Regulatory T-cell
VHL	Von Hippel-Lindau protein
WHO	World Health Organization
ZEB1	Zinc finger E-box binding homeobox 1
2-HG	2-hydroxyglutarate
2-OG	2-oxoglutarate

1 INTRODUCTION

Astrocytomas are tumors of the central nervous system and represent a major subgroup of gliomas. Although they have been intensely studied during the past decades, no potent cure has been found and the prognosis of patients suffering from the disease remains poor. Astrocytomas can be divided into subgroups on the basis of the World Health Organization (WHO) classification of tumors, dividing them in four groups with increasing malignancy. Pilocytic astrocytomas are mainly seen in children and are considered benign due to their non-invasive growing manner. Diffuse astrocytomas, anaplastic astrocytomas and glioblastomas are collectively referred to as diffusely infiltrating astrocytomas. Among these, diffuse astrocytomas are low grade tumors, while anaplastic astrocytomas and glioblastomas are considered to be high grade tumors. The glioblastoma is the most common primary brain tumor and has the worst outcomes in relation to gliomas. Additionally, it is also one of the most severe of all human tumors.

With increasing malignancy, changes occur not only in the tumor cells themselves but also in the tumor microenvironment surrounding these cells. The neoplastic cells express proteins leading to reactions in the tumor surroundings. One of these changes is the awakening of the inflammatory system, including the complement cascade. The inflammatory cells recruited to the extracellular matrix (ECM) release signalling molecules, thus exerting an impact on the direction of tumor progression. The cell's structural connections to its surroundings are loosened due to enzyme secretion and changes in the cellular protein expression. Furthermore, hypoxia occurs when the tumor expands faster than the nutrient and oxygen providing blood vessels grow. All these phenomena ultimately increase the tumor malignancy and tendency to invasiveness.

These phenomena can be studied on the basis of the expression of related molecules, observed by means of immunohistochemistry. C4d can be seen as a marker of an initiated inflammatory reaction. It is the

degradation product of the activated complement factor C4 and labels the site where the complement cascade activation has taken place. Precedently, C4d has been widely studied in allograft rejections, but not in astrocytomas. On the other hand the mutation of the gene encoding the isocitrate dehydrogenase 1 (IDH1) enzyme has already been widely studied in gliomas after its discovery in 2008. It has so far been considered a major milestone in research, in terms of providing an extensive understanding of the process of gliomagenesis and of the differentiation of the glioma types. Constituting a relatively new finding, the manner in which the *IDH1* mutation affects different pathways of protein expression in the glioma cells, and the other alterations it may cause in cell functions, is now under intense research. Expression of the hypoxia-inducible factor 1-alpha (HIF-1alpha) has been shown to be altered in *IDH1* mutated cells. Under hypoxic conditions, HIF-1alpha initiates pathways leading to increased expression of proteins that allow cells to adapt to hypoxia. These include carbonic anhydrase IX (CA IX) and heat shock protein 27 (Hsp27). Expression of these hypoxia biomarker molecules further change properties of the cells, increasing the capability thereof to survive, migrate and invade, among which invasiveness is one of the main problems regarding the treatment of diffusely infiltrating astrocytomas. The epithelial–mesenchymal transition (EMT) is a phenomenon, in which cells transform their phenotype to a more mesenchymal form, enabling the cells to migrate and invade with ease. It has been shown that *IDH1* mutation, HIF-1alpha, Hsp27 and CA IX are all involved in this process. Furthermore, polysialic acid (polySia) is known to loosen the connections between adjacent cells, as well as between cells and the ECM.

Considering that an effective cure to astrocytomas has not been achieved with conventional treatment methods, i.e. radical excision, chemotherapy and radiation therapy, it is clear that new treatment methods are needed. Fundamental research on the oncogenic protein expression pathways can serve as the basis to applied research that can eventually lead to creation of new treatment methods. Hypoxia and inflammation are key elements in tumorigenesis and in the tumor microenvironment. Therefore, it is highly relevant to study astrocytomas from their perspective. EMT along with the increased cell invasion capability are also discussed in this thesis, since these aspects are connected to both hypoxia and inflammation and constitute a

phenomenon of increasing malignancy. The EMT and the proteins related to it could also be potential targets in treatment of astrocytomas in the future.

2 REVIEW OF THE LITERATURE

2.1 Astrocytic tumors

Astrocytic tumors (or astrocytomas) are tumors located in the central nervous system (CNS). Astrocytomas are a subgroup of gliomas, tumors that originate from the supporting tissue of the CNS. Other subgroups of gliomas are roughly divided into oligodendrogliomas and ependymomas. Astrocytomas originate from neural stem cells or their precursors. Astrocytomas can be graded by their microscopic appearance. In practice tumor grading is related to patient prognosis and provides guidelines to patient treatment. The World Health Organization (WHO) classification of CNS tumors divides astrocytomas into four grades (Louis et al., 2007). Four main histological criteria are used in order to assess tumor grading on the basis of the WHO system of 2007. These are atypia, mitosis, microvascular proliferation, and necrosis. The classification system was updated in 2016, adding molecular parameters to the definition of the tumor type and grade (Louis et al., 2016). Yet histological evaluation remains an important aspect in the novel classification. The new classification system is briefly presented later in this chapter. Understandably, the classification of 2007 was used in order to define the tumor material of the studies presented in this thesis. Thus, in the following paragraphs, the characteristic features of different grades of astrocytomas are briefly described on the basis of the WHO classification system by Louis et al. (2007).

Most of the grade I astrocytomas are referred to as pilocytic astrocytomas. They cover approximately 5-6 % of all gliomas, with the overall incidence thereof being 0.37 per 100,000 persons per year. These tumors usually have a clear borderline, are slow growing, and are therefore considered relatively benign. Pilocytic astrocytomas commonly appear in children, and are predominantly located infratentorially in the cerebellum. However, they can form in any region of the neuraxis. Macroscopically pilocytic astrocytomas are often gray in colour and of soft

consistency, with an intratumoral or paratumoral cyst formation. An infiltration of the meninges may take place, however the invasion is not as aggressive as in the case of malignant tumors. Alternately densely organised bipolar cells and a looser cell organisation with microcysts may be observed at a microscopic level. Mitoses are rare. A glomeruloid vascular proliferation may be seen, as in the case of the grade IV glioblastomas. Unlike other astrocytomas, pilocytic astrocytomas have no tendency toward malignant transformation and can hold their histological grade for decades, resulting into a longer survival time of the patient.

Grade II astrocytomas are referred to as diffuse astrocytomas in the WHO classification. These tumors are also generally slow growing. They commonly occur supratentorially in the frontal and temporal lobes, although one can be found in any region of the CNS. Unlike the pilocytic astrocytomas they often proceed into a more malignant grade. Diffuse astrocytomas cover approximately 10-15 % of all astrocytic tumors and the incidence rate thereof is 0.14 new cases per 100,000 persons per year. Most commonly, diffuse astrocytomas first appear in younger adults, with a peak incidence being recorded between the ages of 30-40 years of age. Unlike pilocytic astrocytomas, diffuse astrocytomas lack clear borderlines and grow diffusely infiltrating into the normal brain tissue. Understandably, tumors having such growing manners are virtually impossible to surgically excise completely. A remnant is often left and the tumor reoccurs. Grade III and IV astrocytomas share this feature and thus grade II-IV astrocytomas can be referred to as diffusely infiltrating astrocytomas. Macroscopically diffuse astrocytomas often have a heterogeneous consistence, comprising multiple cysts, and looser and denser tissue areas. Under microscopic observation, they consist of well-differentiated neoplastic astrocytes often with microcystic surroundings. Cellularity is increased; however mitoses do not exist or are rare.

Anaplastic astrocytomas are WHO grade III tumors. They are considered to be high grade and malignant astrocytomas. The annual incidence rate is 0.37 new cases per 100,000 persons. Anaplastic astrocytomas most often occur in the cerebral hemispheres of adult patients, the average age being 45-50 years. Macroscopically, the anaplastic astrocytoma often resembles the diffuse astrocytoma and it can be difficult to discriminate between the two. Histologically, nuclear atypia, increased cellularity and mitoses are apparent. Anaplastic astrocytomas

may be viewed as an intermediate form of an astrocytoma transforming from a diffuse astrocytoma to a glioblastoma. However, it is possible for an anaplastic astrocytoma to develop de novo, without a previous lower grade lesion. In general, anaplastic astrocytomas have a strong tendency to proceed into a grade IV astrocytoma.

Glioblastoma multiforme (GBM, also often abbreviated to mere glioblastoma) are the most aggressive astrocytomas and are classified to WHO grade IV tumors. Unfortunately, GBMs are also the most frequent form of astrocytomas and primary brain tumors. GBMs account for approximately 60-75 % of astrocytic tumors and 12-15 % of all primary brain tumors. In most western countries the incidence is 3-4 new cases per 100,000 persons per year. It most commonly occurs in patients between 45 and 75 years of age, being located in the subcortical white matter of a cerebral hemisphere. The growing pattern is extremely aggressive including destruction of normal brain tissue. The tumor often spreads rapidly, possibly extending even to the contralateral hemisphere over the corpus callosum. The invasion proceeds intracranially. Extracranial spreading or metastasing is extremely uncommon, as is the case in all astrocytomas.

GBMs can be divided into primary and secondary GBMs. Primary GBMs occur de novo, without a pre-existing lower grade astrocytoma, whereas secondary GBMs develop via the malignant progression of grade II and III astrocytomas. Primary GBMs constitute 90 % of all GBMs. The transformation of a diffuse astrocytoma into a secondary GBM has been estimated to endure from one to over ten years, with a mean interval of approximately 4-5 years. The histological features of GBMs are nuclear atypia, mitoses, endothelial proliferation and necrosis. Macroscopically, these can be seen as necrotic foci and haemorrhage. The GBM has several histological variants, including gliosarcoma and giant cell glioblastoma.

2.1.1 Genetics of astrocytic tumors

The isocitrate dehydrogenase 1 (*IDH1*) mutation is considered to be the main hallmark of low-grade astrocytomas (Ichimura et al., 2015). *IDH1* mutation occurs in approximately 80 % of grade II-III astrocytomas and in secondary GBMs, but is rare in primary GBMs (Balss et al., 2008; Ohgaki and Kleihues, 2009; Yan et al., 2009; Mellai et al., 2011). *IDH1*

mutation is an early genetic change in gliomagenesis. It appears to occur even earlier than the *TP53* mutation (Watanabe et al., 2009). After the discovery of the *IDH1* mutation, it has been suggested that primary and secondary GBMs could after all originate from different types of progenitor cells, despite their histological resemblance (Ohgaki and Kleihues, 2009).

The *TP53* mutation and an increased expression of PDGFR (platelet driven growth factor receptor) are also early events in the gliomagenesis of grade II and III astrocytomas and secondary glioblastomas. The *TP53* mutation occurs in more than 60 % of these tumors and can also be considered as a hallmark of low-grade astrocytomas. In low-grade astrocytomas *TP53* mutation can be considered as a prognostic marker for shorter survival (Ohgaki and Kleihues, 2011).

ATRX mutation is another gene mutation commonly seen in diffuse and anaplastic astrocytomas. It is frequently associated with *IDH1* mutation (Ichimura et al., 2015). ATRX is an enzyme involved in chromatin remodelling and in the alternative lengthening of telomerases. The mutation is almost exclusive to diffuse and anaplastic astrocytomas and is rarely seen in GBMs (Jiao et al., 2012).

Regarding glioblastomas, there are different frequencies in the genetic alterations seen in primary and secondary GBMs. Genetic alterations that are significantly more frequent in primary GBMs than in secondary GBMs are the loss of heterozygosity (LOH) 10p (in 70 % of cases), EGFR amplification (in 36 % of cases), p16(INK4a) deletion (in 31 % of cases) and *PTEN* mutations (in 25 % of cases). Significantly more frequent alterations in secondary GBMs include *TP53* mutations (in 60 % of cases), LOH 19q and LOH 22q (Ohgaki and Kleihues, 2007; Benito et al., 2010; Ohgaki and Kleihues, 2011).

The biologic background of pilocytic astrocytomas differs extensively from higher grade astrocytomas. They rarely express any of the changes presented above regarding the diffusely infiltrating astrocytomas. A common mutation in pilocytic astrocytomas is a KIAA1549-BRAF fusion gene, resulting from the duplication of 7q34 (Forshew et al., 2009). This causes alterations in the ERK/MAP kinase pathway, increasing transcriptional activity and cellular proliferation. Furthermore, the genetics of diffusely infiltrating astrocytomas found in children differ from those found in adult patients and do not possess *IDH1* or *TP53* mutations,

but instead are often *BRAF* mutated or display structural alterations involving *MYB/MYBLI* or *FGFR1* mutations (Ichimura et al., 2015).

As presented in the beginning of this chapter, the WHO classification system of gliomas was remodelled in 2016. The classification now underlines the molecular and genetic characterisation of the tumors beside by histology. Precedently, gliomas were divided into subgroups of astrocytomas, oligodendrogliomas, oligoastrocytomas and ependymomas. Now a new umbrella concept of diffuse gliomas has been introduced. The diffuse glioma category includes grade II-IV astrocytomas, grade II and III oligodendrogliomas, grade II and III oligoastrocytomas, and the related diffuse gliomas. The main reason behind this new categorisation is the recovery of the *IDH* mutation, which is a shared trait of these tumors. The nomenclature of astrocytoma grades remains the same as in the classification of 2007, yet they are further divided into subgroups by the *IDH1* or *IDH2* mutation status as either mutated or wild-type tumors. Moreover, a main change in the classification system is the 1p/19q codeletion to be named specifically in relation to oligodendrogliomas. Precedently, a loss at 1p/19q was thought to be predominant in oligodendrogliomas, but also possible in astrocytomas (approximately in 15 % of diffuse astrocytomas and in 70 % of oligodendrogliomas (Ohgaki and Kleihues, 2011)). Thus, upon applying the new classification all tumors showing a 1p/19q codeletion are classified as oligodendrogliomas.

2.1.2 Clinical aspects

Astrocytomas are the most common type of primary brain tumors. Yet they are considered particularly challenging to treat and in the case of all astrocytic tumors prognosis is rather poor when compared to other tumors, excluding pilocytic astrocytomas. The understanding of the biological mechanisms involved in gliomagenesis and glioma phenotypes has been greatly expanded during the past two decades, yet no effective cure has been found to significantly improve the long-term survival. Patients with diffuse astrocytoma typically survive for more than 5 years, while patients with anaplastic astrocytoma survive for 2–3 years on average, whereas the majority of GBM patients die to the disease within 1 year (Louis et al., 2007; Komori, 2015). Unfortunately, glioblastoma is the

most common astrocytoma, and also the most common form of all primary brain tumors.

In each case, a combination of WHO grade and clinical parameters, such as the age of the patient, performance status, and extent of surgical resection, contributes to the overall estimate of the prognosis and makes it possible to determine the form of therapy (Komori, 2015). The most common treatment procedure is the maximal safe resection of the tumor, followed by radiotherapy with post-radiotherapy temozolomide chemotherapy. Patients with pilocytic astrocytomas can often be cured with resection of the tumor alone, due to the non-invasive growing pattern of the tumor (Louis et al., 2007; Bonfield and Steinbok, 2015). Furthermore, in the case of anaplastic astrocytomas a mere total resection may be chosen for treatment. High grade gliomas consist of a core mass and of invasive neoplastic cells infiltrating the normal brain tissue centimetres away from the tumor (Giese et al., 2003). Thus, it is highly common that residual cancer cells are left upon completion of surgery and that the tumor recurs.

2.2 C4d and complement activation

The complement is a component of the innate immune system. The complement cascade can be initiated via three different pathways, which are the classical pathway, alternative pathway and lectin pathway (Trouw and Daha, 2011). The classical and lectin pathways are known to involve C4d (Murata and Baldwin, 2009). Activation of the classical pathway of complement requires an antigen-antibody complex, whereas the lectin pathway can be activated by C3 hydrolysis or plain antigens, in the absence of antibodies. The complement is initiated upon the activation of C1, leading to formation of a C3 convertase and to the release of C3a and C3b. After activation and splitting of C3, the complement cascade further proceeds by cleaving the next zymogen of the cascade into a functioning enzyme. C4d is the degradation product of activated complement factor C4. Complement control protein factor I inactivates cascade protein C4b decomposing it into C4c and C4d (Baldwin et al., 2004). This way an activated complement cascade leaves behind inactive fragments of the complement components, including C4d. C4d binds covalently to the

activation point. It appears that C4d itself has no function in the immune system, but it can be considered as a 'footprint' of activation of the immune defence since it binds covalently to the activation point and it has a relatively long half-life (Nickeleit and Mihatsch, 2003). The end results of the complement cascade are the membrane attack complex (MAC), protein opsonization, inflammation and increased vascular permeability. MAC injures cell membrane and causes osmotic lysis of the cell. The complement promotes inflammation by attracting neutrophils, eosinophils, basophils, macrophages, monocytes, and T cells to the activation site via the complement fractions C5a and C3a (Guo and Ward, 2005; Kumar et al., 2010). Increased vascular permeability is due to histamine being released by the mast cells, which are activated by C3a and C5a. Figure 1 shows how the activation of the complement cascade is triggered and the effector functions of follow through the system.

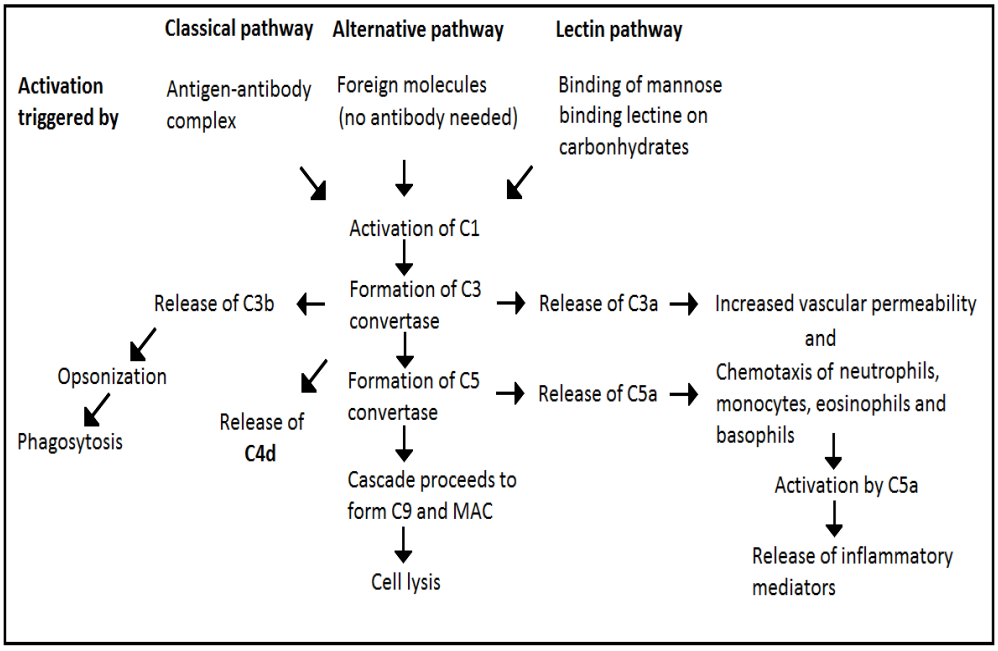

Figure 1. Activation of the complement pathways and the effector functions of the complement system (Kumar et al., 2010). MAC: Membrane attack complex

In practice, C4d is mainly connected to allograft tissue rejection and has been widely studied particularly in kidney allografts. In this sense, C4d is currently used as a marker for the antibody mediated rejection observed

in the microvasculature of the kidney allograft (Colvin, 2009). Recent surveys concerning pregnancy and systemic autoimmune diseases have also taken notice of C4d as a potential biomarker bringing out harmful antibody activation (Cohen et al., 2012). In allograft studies, the activation of C4d has been predominantly observed at the level of the endothelial cells, but it has also been present in the tumor neoplastic cells themselves. Additionally, their adjacent extracellular matrix can express C4d (Lucas et al., 1996; Bu et al., 2007). Lucas et al. (1996) found C4d immunostaining in the cells of the papillary thyroid carcinomas, whereas Bu et al. (2007) found C4d in the proximity of the neoplastic follicular dendritic cells of follicular lymphomas. The extent of research conducted on C4d in the CNS and its tumors is scarce.

It has been commonly thought that the immune system prevents cancer formation by destroying damaged and abnormal cells. However the theory of immunoediting describes a wider process, explaining how the immune system finally becomes inefficient against tumor formation (Dunn et al., 2002). In this theory, the immune system initially destroys the neoplastic cells but subsequently becomes inefficient. At first, the immune system specifically recognises antigens that originate from mutations in the malignant cells (Schumacher and Schreiber, 2015). However, during malignant progression, the cells undergo further mutations that affect the capability of the immune system to control tumor growth. Immunoevasion, or in other words capability to evade immune destruction, is finally accomplished by the malignant cells. Yet the inflammation continues, forming an inflammatory microenvironment, which is associated with cancer progression (Hanahan and Weinberg, 2011).

In a normal brain, the blood brain barrier limits the entry of inflammatory cells. Nevertheless, in a diseased state, the integrity of the barrier is damaged, thus enabling the immune cells to migrate past the barrier (Engelhardt and Ransohoff, 2012). Inflammatory cells are recruited to the site. It seems that the inflammatory microenvironment causes a change in the phenotype of the inflammatory cells (Noy and Pollard, 2014). The polarised inflammatory cells are in turn involved in the malignant progression. They release growth factors (such as the tumor growth factor EGF and angiogenic growth factor VEGF), proangiogenic factor FGF2, chemokines (CC- and CXC- receptors), cytokines (TGF-β,

TNF-α, IL-6, IL-1β), and ECM modifying enzymes (Murdoch et al., 2008; Qian and Pollard, 2010; Elinav et al., 2013). These in turn release cells to migration and lead the cells towards a malignant progression. Active inflammatory cells also release oxygen species that are mutagenic to nearby cells, thus increasing the possibility of DNA mutations (Mantovani et al., 2008; Fiaschi and Chiarugi, 2012). Thus, it seems that the inflammatory cells and the neoplastic cells are almost in dialogue via signalling molecules, subsequently leading to the formation of an unfavourable tumor microenvironment.

2.3 Isocitrate dehydrogenase 1

Isocitrate dehydrogenase 1 (IDH1) is an NADP+ dependent enzyme that catalyses the oxidative decarboxylation of isocitrate in order to produce alpha-ketoglutarate (alpha-KG) (Losman and Kaelin, 2013). Alpha-ketoglutarate is also known as 2-oxoglutarate (2-OG) (Huergo and Dixon, 2015). Besides IDH1, human cells express IDH2 and IDH3. The enzymes are isoform with each other, yet IDH1 is cytoplasmic, whereas IDH2 and IDH3 function in mitochondria (Losman and Kaelin, 2013).

The *IDH1* mutation, or the mutation of the gene encoding the IDH1 enzyme, was originally discovered in GBMs (Parsons et al., 2008). As previously demonstrated, the mutation of the gene encoding *IDH1* is common in grade II and III astrocytomas as well as in secondary GBMs. The *IDH1* gene is located on chromosome 2q33.3 (Liu and Ling., 2015). *IDH1R132* and *IDH1R172* are the most common mutation sites in gliomas, the predominant amino acid sequence alteration being R132H and accounting for approximately 90 % of the *IDH1* mutations in gliomas (Hartmann et al., 2009).

When the *IDH1* gene is mutated, the IDH1 enzyme reduces alpha-KG to 2-hydroxyglutarate (2-HG) (Dang et al., 2009). This causes other changes to the cell's properties. High levels of 2-HG accumulate in the tumor cells and affect various signalling pathways which regulate cell proliferation and differentiation. First of all, it appears that 2-HG competitively inhibits alpha-KG dependent enzymes, among which many regulate the expression of other molecules that assume different biological functions in the cell (Loenarz and Schofield, 2008; Xu et al., 2011; Losman

and Kaelin, 2013). Therefore, the stabilisation of several tumor related protein expression pathways may be altered because of *IDH1* mutation mediated depletion of alpha-KG or because of direct inhibition of related enzymes by 2-HG.

Secondly *IDH1* mutation has been shown to alter DNA's histone methylation, which once more exerts an influence on the expression of molecules and on the epigenetic properties of DNA (Xu et al., 2011; Lu et al., 2012; Venneti and Thompson, 2013). Lu et al. (2012) suggest that the increased histone methylation is due to the inhibition of the histone demethylase enzymes by 2-HG. Additionally, DNA hypermethylation has been detected in *IDH1* mutated astrocytes when compared to similar cells with wild-type *IDH1* gene, affecting again the epigenetic regulation (Turkan et al., 2012). Turkan et al. (2012) showed that both the production of 2-HG and the levels of α-KG can affect the DNA methylation status. They also showed that the *IDH1* mutation is associated with the CpG island methylator phenotype (CIMP), characterised by extensive hypermethylation at specific locations of the DNA. One typically hypermethylated gene in the CIMP is the gene encoding O-6-methylguanine DNA methyltransferase (MGMT). Methylation of MGMT favourably affects patient prognosis and improves response to temozolomide therapy in GBMs (Hegi et al., 2005).

Although it seems that *IDH1* mutation is tightly involved in gliomagenesis, it has been widely shown that it affects patient prognosis in a favourable manner (Beiko et al., 2014; Houillier et al., 2010; SongTao et al., 2012), i.e. patients with an *IDH1* mutated diffusely infiltrating astrocytoma have better prognosis than patients with an *IDH1* non-mutated tumor.

2.4 Heat shock protein 27

Heat shock proteins (HSP's) are a family of proteins that act as molecular chaperones in protein folding and aid proteins to form their normal structure. Thus, they prevent creation of nonspecific and deformed proteins. According to their size, heat shock proteins have been classified into subgroups, namely Hsp40, Hsp60, Hsp70, Hsp90 and small HSPs, including Hsp27 (also known as HSPB1).

At first expression of Hsp27 was shown to be increased by sudden rise of temperature in the cell, hence the name. Later studies have shown that many other cell stressing conditions can upgrade the expression of Hsp27, such as hypoxia, oxidative stress, ischemia and exposure to toxic radicals and carcinogens (Yu et al., 2008; Khalil et al., 2011; Marotta et al., 2011). Similarly to other heat shock proteins, Hsp27 also assumes a cytoprotective role in the cell. Besides facilitating protein folding, Hsp27 is involved in the proteasome mediated breakdown of cytotoxic proteins (Parcellier et al., 2003). In the presence of oxidative stress, Hsp27 functions as an antioxidant. It lowers the levels of the reactive oxygen species by upholding intracellular glutathione in its reduced form and by lowering the levels of intracellular iron (Arrigo et al., 2005). The functions of Hsp27 can also prevent apoptosis in cells that are in unfavourable conditions and would normally proceed into apoptosis (Concannon et al., 2001).

An overexpression of Hsp27 has been detected in various cancer types. Altogether, the expression of heat shock proteins in neoplastic cells is essential to many of their typical features, including uncontrolled proliferation, decreased tumor suppression, advanced cell survival, as well as angiogenic and metastatic properties (Calderwood and Gong, 2016). Distinctively in astrocytomas, Hsp27 has been connected to tumor malignancy and poor differentiation as well as with the Ki-67/MIB-1 proliferation labelling index (Khalid et al., 1995; Hermisson et al., 2000; Assimakopoulou and Varakis, 2001; Shen et al., 2010). Although beneficial in normal tissues, the cytoprotective Hsp27 activity in cancer cells is disadvantageous from the patient's point of view, since it is connected to the tumor promoting adjustments in cells, as presented above. In this manner, the Hsp27 activity in cancer cells can promote cancer progression. This has also been demonstrated in practice, considering that in clinical research Hsp27 expression has been connected to resistance to chemotherapy (Calderwood and Ciocca, 2008). It has been suggested that avoiding apoptosis is the key mechanism in cancer resistance to treatment (Giese et al., 2003; Khalil et al., 2011). In addition, the epithelial-mesenchymal transition (EMT) has been lately connected to invasion and metastasis. It has been shown that Hsp27 is involved in the EGF-mediated epithelial to mesenchymal transition, by way of

modulation of the beta-catenin/Slug signalling pathway (Shiota et al., 2013; Cordonnier et al., 2015).

2.5 Carbonic anhydrase IX

Carbonic anhydrases (CAs) are zinc containing metalloenzymes that catalyse the reversible reaction in which the carbon dioxide is hydrated in order to obtain a bicarbonate molecule and a proton. They are involved in physiological processes, such as controlling the acid-base balance of tissues, respiration, ion transport and bone resorption (Adeva-Andany et al., 2015). CA IX is mainly expressed in neoplastic tissue and is rarely expressed in normal human tissues (Supuran and Winum, 2015). Expression of CA IX is strongly upregulated under hypoxic conditions via the hypoxia-inducible factor 1 (HIF-1) pathway (Rademakers et al., 2008).

In gliomas, CA IX positivity has been found to associate with increasing malignancy of the WHO grade in diffusely infiltrating astrocytomas and to have a prognostic value in patient survival, having an unfavourable impact on the latter (Haapasalo et al., 2006). Furthermore, in the case of GBMs the overexpression of CA IX has been connected to poor patient survival and resistance to therapy (Proescholdt et al., 2012).

The consequences of CA IX activity in cells resemble the ones of Hsp27 and are also unbeneficial from the patient's point of view. In neoplastic tissues, the overexpression of CA IX has been connected to an increased capability of tumor cells to survive, migrate and invade (Svastova et al., 2012; Svastova and Pastorekova, 2013). Again, these phenomena can be explained by the microenvironmental changes caused by the functioning of CA IX. In hypoxia, the cell metabolism turns into anaerobic glycolysis in which lactic acid is produced and intracellular pH is lowered. CA IX activity allows the cell to restore pH homeostasis, although the pH of the ECM is lowered in this instance. This results in two cancer progressing phenomena: firstly the stabilisation of the pH facilitates tumor cell survival, and secondly a decrease in the ECM pH facilitates tumor cell migration and invasion. Acidosis in ECM activates particular enzymes which break down the ECM architecture, making it easier for the tumor cells to invade surrounding tissues (Parks et al., 2011; Svastova and

Pastorekova, 2013). Additionally, CA IX is involved in EMT, and in several other mechanisms that facilitate cell migration (Svastova et al., 2012).

2.6 Hypoxia-inducible factor 1-alpha

Hypoxia develops in fast growing tumors when the vasculature becomes insufficient in order to measure up the needs of the tumor's oxygen requirement. Hypoxia-inducible factor 1(HIF-1) is a transcription factor that is expressed in cells exposed to hypoxic conditions (Semenza, 2013). The main function of HIF-1 is to regulate expression of other molecules that help cells adapt to hypoxia (Kaluz et al., 2008). HIF-1 is heterodimer composed of two subunits, alpha and beta. HIF-1beta is expressed in cells regardless of normoxia. Contrary to the beta subunit, the HIF-1alpha expression is highly sensitive to lowered oxygen concentrations. HIF-1alpha regulated genes are partly responsible for tumor growth, survival and migration under hypoxic conditions, and also predominantly responsible for tumor resistance against radiation therapy and chemotherapy (Pouysségur et al., 2006; Said et al., 2010).

As presented in the previous chapters, Hsp27 and CA IX are both molecules that help hypoxic cells to adapt to lowered oxygen levels. HIF-1alpha is a transcription factor of both Hsp27 and CA IX (Wykoff et al., 2000; Proescholdt et al., 2005; Whitlock et al., 2005; Said et al., 2010; Semenza, 2010; de Thonel et al., 2012). Activation of the HIF-1alpha pathway is controlled by the Von Hippel-Lindau protein (VHL) (Robinson and Ohh, 2014). In normoxia, HIF-1alpha undergoes proline hydroxylation by means of alpha-KG dependent propyl-4-hydroxylases (PHD) (Schofield and Ratcliffe, 2005) and the VHL binds to the hydroxylated HIF-1alpha. The molecule is subsequently ubiquitinated and broken down. If there is oxygen deprivation in the cell, HIF-1alpha is not hydroxylated and the VHL does not recognise it. HIF-1alpha is stabilised, translocated to the cell nucleus and dimerised with HIF-1beta. This pathway results in an active transcription factor which can in turn launch the pathways of other molecules that help the cell to adapt to hypoxia, such as Hsp27 and CA IX. Another factor mediating the HIF pathway, is the factor inhibiting HIF (FIH), which is also an alpha-KG dependent oxygenase (Rodriguez et al., 2016; Taabazuing et al., 2016), that interacts

with HIF-1alpha and the VHL in order to mediate repression of transcriptional activity of HIF-1alpha (Mahon et al., 2001).

2.7 Neural cell adhesion molecule and polysialic acid

The neural cell adhesion molecule (NCAM) is a cell adhesion molecule which mediates the adhesion between adjacent neurons and glial cells, as well as the ECM (Seifert et al., 2012; Dallérac et al., 2013). There are three isoforms of NCAM, among which two are transmembrane molecules and the third is attached to the cell membrane by means of a glycosylphosphatidylinositole anchor and has no intracellular domain (Walmod et al., 2004). NCAM is found in most tissues in humans, but most frequently in nervous tissues, including both central and peripheral nervous tissues.

The polysialic acid (polySia) is a carbohydrate polymer. It is added to the extracellular part of the NCAM post-translationally, being subsequently located on the cell surface (Finne et al., 1983). In normal tissues, particularly during embryonic development, polySia is essential in neuronal cell migration and axon pathfinding (Brusés and Rutishauser, 2001). Polysialylated NCAM (polySia-NCAM) is considered to be a neural stem cell marker (Pennartz et al., 2004). PolySia is widely hydrated and strongly negatively charged. It spans the hydrodynamic area of the cells extern proximity and weakens NCAM's capability to maintain the cell adhesion structures (Rutishauser, 1998; Seifert et al., 2012). In relation to the solidity of the tissue architecture, it has been suggested that NCAM alone and polysialylated NCAM (polySia-NCAM) play opposite roles; NCAM stabilises cell adhesion, whereas polySia-NCAM facilitates cell migration and plasticity as well as increases the cell's invasion capability in gliomas (Rutishauser and Landmesser, 1996; Suzuki et al., 2005). In clinical trials, expression of polySia has been mainly associated with an increasing WHO grade in astrocytomas (Petridis et al., 2009; Amoureux et al., 2010). Conversely, NCAM expression has been shown to correlate with a lower malignancy grade (Todaro et al., 2007; Duenisch et al., 2011). Amoureux et al. (2010) and Haque et al. (2011) suggest that polySia-NCAM is a biomarker of patient prognosis in gliomas, indicating an unfavourable outcome.

Although it seems that both polySia and NCAM are associated to EMT, previous studies have mainly shown that polySia and NCAM associated EMT targets E-cadherin connections, causing the loss of E-cadherin mediated connections (Frame and Inman, 2008; Lehembre et al., 2008; Schreiber et al., 2008). E-cadherin rarely occurs in gliomas (Iwadate, 2016). Thus, it could be that polySia is not involved in the EMT in the case of gliomas by changing the cell morphology, but it is likely to mechanically loosen the cell connections to the surrounding structures, thus becoming subsequently involved in cell migration. Furthermore, NCAM expression has been shown to correlate with tumor invasion (Lehembre et al., 2008).

2.8 Hypoxia, inflammation and epithelial-mesenchymal transition

Neoplastic cells are surrounded by the ECM, in which the tumor microenvironment is formed. The neoplastic cells and other ECM infiltrated cells create the tumor microenvironment by secreting growth factors and cytokines and by modulating the hydration and pH homeostasis, as well as function of adhesion molecules (Pickup et al., 2014). The types of cells that contribute to the formation of the tumor microenvironment via signaling molecules (in addition to cancer cells) are cancer stem cells, endothelial cells, pericytes, cancer associated fibroblasts and immune inflammatory cells (Hanahan and Winberg, 2011; Hanahan and Coussens, 2012). These cells of the tumor microenvironment contribute to the acquisition of the core cancer hallmarks (Hanahan and Coussens, 2012).

Hanahan and Weinberg have presented the hallmarks of cancer that constitute common traits of the cancer cells, and are obtained during the multistep development of tumors (Hanahan and Weinberg, 2011). They include “sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis”. They have also presented emerging hallmarks that constitute phenomena characteristic to cancer, but their complete mechanisms in the formation and progression of cancer are yet to be fully understood. The emerging hallmarks are “evading immune destruction and deregulating cellular energetics”. As enabling characteristics for both core and emerging

hallmarks they refer to “genome instability and tumor promoting inflammation”. In the chapter illustrating the role of C4d, the functions of the inflammatory microenvironment were already briefly described. However, the effects of the polarised inflammatory cells are wide and are further elaborated in the next chapters, elucidating importance of the enabling hallmark of the tumor promoting inflammation. It can also be noted that *IDH1* mutation is triggered by genome instability, the other cancer enabling hallmark.

Besides the inflammatory cells, hypoxia in the tumor microenvironment has an essential role in acquisition of the cancer hallmarks. Hypoxia causes a specific gene expression in the cells and changes in the tumor microenvironment. Hypoxia is a remarkable cell stressor that induces the adaptations essential for the survival and metastatic abilities of the neoplastic cells. Gained adaptive mechanisms include angiogenesis, altered metabolism, epithelial-mesenchymal transition (EMT), invasion capability, a decreased response to the immune system as well as resistance to chemotherapy and radiation therapy (Gillies and Gatenby, 2007; Hanahan and Weinberg, 2011; Lendahl et al., 2009; Gillies et al., 2012). Furthermore, these pathological changes cause additional alterations in the gene expression and changes in the cells’ properties, leading to an increase in the tumor malignancy.

In the case of the EMT, the epithelial cells assume a mesenchymal form. The mechanism is essential for the development of embryos and in wound healing, but it is also a phenomenon associated with cancer, increasing the migration, invasion and metastasis of the cancer cells (Kalluri and Weinberg, 2009). The formation results in a loss of the cell to cell and cell to ECM adhesions. The transformed cells also express ECM degrading enzymes, and have an increased motility and resistance to apoptosis. This way the transformation enables cells to migrate and disseminate (Hay, 1995; Hanahan and Winberg, 2011).

The most important cell to cell contacting adhesion molecule involved in the EMT is E-cadherin. However, the E-cadherin expression in gliomas is rare, yet factors that induce EMT via E-cadherin in other cancers can also activate the EMT in gliomas (Iwadate, 2016). In other cancers the EMT inducing factors mostly target the E-cadherin expression, but as previously indicated, in the case of gliomas these inducing factors initiate the EMT via other mechanisms, independently of E-cadherin. For

example, Twist is an EMT initiation factor protein upregulated in malignant gliomas, which promotes cell invasion via the mesenchymal target gene Slug (likewise Hsp27) and the fibroblast activation protein, independently of E-cadherin (Mikheeva et al., 2010; Nordfors et al., 2015; Iwadate, 2016).

However, hypoxia and inflammation are the most common activators of the EMT in gliomas and can launch the transition directly (Yi et al., 2011; Ye et al., 2012). In the case of inflammation and hypoxia, tumor associated macrophages/microglia (TAM/Ms), are drawn to tumor stroma. In neoplastic tissues, macrophages polarise to tumor associated macrophages (Sica et al., 2008; Gabrilovich et al., 2012; Mantovani et al., 2012). TAMs constitute the largest population of stromal cells in tumors (McDonald et al., 2016). The TAM/Ms release growth factors including tumor growth factor-beta (TGF-beta), which in turn triggers alterations in DNA's transcription factors required to initiate the EMT (Zhang et al., 2016). It has also been shown that TGF-beta can induce expression of HIF-1alpha.

In severe hypoxia, necrosis i.e. unprogrammed cell death occurs. In necrosis cells become bloated and disintegrate, generating cellular debris that must be cleared by macrophages. Activation of the complement cascade releases the anaphylatoxins C5a and C3a, which attract macrophages, eosinophils, monocytes, and T cells to the inflammation site (Guo and Ward, 2005). Yet again, tumor microenvironment shifts macrophages towards a tumor-promoting phenotype. Besides EMT, TAM/Ms in the tumor microenvironment have been shown to associate with tumor progression, tumor cell growth, angiogenesis and immunosuppression (Sica et al., 2008; Qian and Pollard, 2010; Magaña-Maldonado et al., 2016).

Under hypoxic conditions, the most important inflammatory cells involved in the malignant transformation, besides TAM/Ms, are the myeloid-derived suppressor cells (MDSC) and regulatory T-cells (Treg) (McDonald et al., 2016). The MDSC are induced by tumor secreted growth factors, yet the MDSC secrete immunosuppressive cytokines (Wesolowski et al., 2013). Treg are involved in angiogenesis via chemokine CC-chemokine ligand 28 and also in tumor immunoevasion (Facciabene et al., 2011; Facciabene et al., 2012).

In GBMs, immunosuppression and immunoevasion are enabled by production of immunosuppressive cytokines, inhibition of T cell proliferation and effector responses, activation of Treg, as well as tissue hypoxia (Razavi et al., 2016). T cell activation is blocked by TGF-beta which promotes immunosuppression, suppresses natural killer cell activity and promotes Treg (Fontana et al., 1991).

Treg promoting factors are programmed cell death 1 (PD-1), which is a transmembrane receptor found on T lymphocytes and its ligands, Programmed cell death ligand 1 (PD-L1, also known as CD274 or B7-H1) and Programmed death ligand 2 (PD-L2, also known as CD273 or B7-DC). PD-1 is found mainly on T cells, Treg, B cells, activated monocytes, macrophages, dendritic cells and natural killer cells (Inaguma et al., 2016; Li et al. 2016a). Binding of PD-L1 to PD-1 downregulates the immune system by promoting apoptosis of antigen specific T-cells, and by activating Treg and decreasing the rate of their apoptosis. Thus, the PD-1 and PD-L1 act as an immune checkpoint downregulating the immune system. The main physiological purpose of the system is to maintain an immune homeostasis and self-tolerance in order to prevent autoimmunity (Francisco et al., 2010). Expression of PD-1/PD-L1 is upregulated in various types of cancer (Inaguma et al., 2016). Furthermore, in the case of GBMs PD-L1 is overexpressed and it has been shown to unfavourably affect patient prognosis (Nduom et al., 2016). The overexpression has been connected to the core cancer hallmark of immune evasion.

Finally, Grassian et al. showed that *IDH1* mutation is associated with EMT via accumulation of 2-HG (Grassian et al., 2012). They demonstrated that high levels of 2-HG cause an EMT-like phenotype, exhibiting changes in EMT-related gene expression and cellular morphology. They also showed that EMT induced by *IDH1* mutation depends on up-regulation of the transcription factor ZEB1 and on down-regulation of the miR-200 family of microRNAs. Up-requlation of ZEB1 is also considered an EMT-inducing signal in gliomas (Iwadate, 2015).

As presented above, C4d, HIF-1alpha, Hsp27, CA IX, polySia as well as *IDH1* mutation all relate to EMT and cell migration and invasion in a rather complex and overlapping manner. The pathways leading to EMT explained above are also demonstrated in Figure 2.

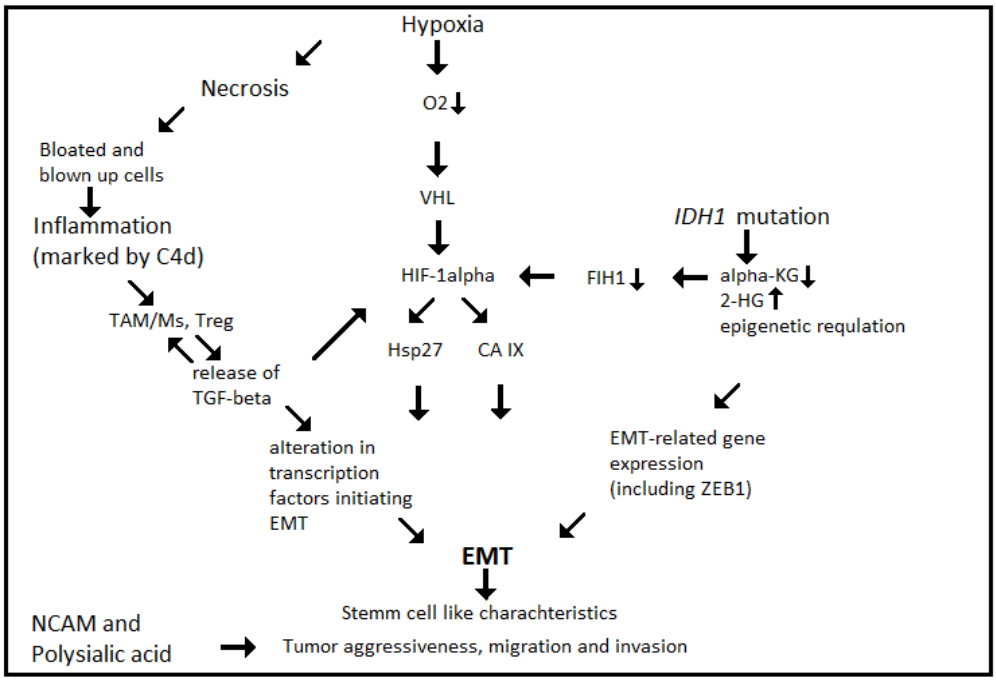

Figure 2. The figure demonstrates how the *IDH1* mutation and expression of C4d, Hsp27, CA IX, HIF-1alpha, NCAM and polysialic acid are involved in the epithelial-mesenchymal transition and in the progression of tumor malignancy in gliomas. Alpha-KG: alpha-ketoglutarate, CA IX: carbonic anhydrase IX, EMT: epithelial-mesenchymal transition, FIH: factor inhibiting HIF, HIF-1alpha: hypoxia-inducible factor 1-alpha, Hsp27: heat shock protein 27, TAM/Ms: tumor associated macrophages/microglia, VHL: Von Hippel-Lindau protein, 2-HG: 2-hydroxyglutarate

3 AIMS OF THE STUDY

The gene expression and cell functions of neoplastic cells vary widely during the multistep development of tumors. In practice, this is indicated by the altered expression of different molecules that are overexpressed in tumors as opposed to normal tissues. A practical way to detect the presence of these molecules is represented by the immunohistochemical staining and evaluation of the staining patterns and extent by means of microscopy. This way important information pertaining to the tumor characteristics is gained, that can subsequently direct the decisions on treatment and help predict patient prognosis.

However, many of potential marker molecules have not yet been found or studied. Within the framework of this thesis, known oncogenic molecules were selected, which are not yet comprehensively studied in astrocytomas, but are connected to other tumors or other pathological conditions. These include C4d, Hsp27, polySia and NCAM.

The first aim was to explore whether an overexpression of these molecules may be identified in astrocytomas, their expression patterns and how they relate to clinicopathological features (such as the WHO grade and patient survival).

The second aim was to preliminary study the expression pathways by investigating the correlations between the expression of the studied molecules and the *IDH1* mutation. The expression of HIF-1alpha and CA IX was also evaluated in order to study the hypoxia-driven expression pathway of Hsp27 and CA IX.

The third aim was to provide a basis for applied research focused on finding new potent treatments for astrocytic tumors.

The fourth aim was to determine whether the expression of the presented molecules is grade specific in astrocytic tumors. The morphological diagnosis and grading of astrocytomas could also become easier, if new grade specifically overexpressed molecules were identified.

4 MATERIALS AND METHODS

4.1 Gathering the study material

Tumor samples were obtained from surgically operated patients at the Tampere University Hospital, Tampere, Finland, during the period 1983-2001. Surgery was performed as part of treatment, and accomplishment of the studies did not affect the treatments selected and administered to the patients. Tumors were removed using the highest level of safe resection. For survival data and other clinicopathological features, patients were monitored after the tumor resection until 2012 or until they passed away. Patient information, such as age, sex, or the number of the resection, radiotherapy and chemotherapy given were gathered and included into the data. None of the patients received temozolomide treatment, due to the limited period of time during which the study material was gathered. An update to the data was performed in 2012 and therefore the study material varied in the first and two subsequent studies. This update mainly touched the survival data. Also because of the study period, the WHO classification system of tumors from year 2007 was used in order to define the tumor grade.
The study protocol of all studies I-III was approved by the Ethical Committee of the Tampere University Hospital and the National Authority for Medicolegal Affairs in Finland.

4.1.1 Patient material in study I

The study material consisted of 102 astrocytomas, of which 9 were grade I pilocytic astrocytomas and 93 were grade II-IV diffusely infiltrating astrocytomas (grade II: 21; grade III: 16; grade IV: 56). Of the 93 diffusely infiltrating astrocytomas, 67 were primary tumors and 26 were recurrent. The mean patient age was 59 years, the youngest patient being 12 years old

and the oldest 85 years old. Overall survival rates were known for 62 patients.

4.1.2 Patient material in study II

The study material consisted of 295 diffusely infiltrating astrocytomas (grade II: 43; grade III: 31; grade IV: 221). There were 264 patients with primary astrocytomas and 31 patients with recurrences only. The data related to the administered radiotherapy and chemotherapy was known for 275 patients. Among these patients, 87 received only radiotherapy and 4 received only chemotherapy. A total number of 59 patients received both radio- and chemotherapy. An additional number of 125 patients were treated with mere surgical resection. The overall survival was known for 247 patients. The mean patient age was 63 years; the youngest patient was 20 years old, and the oldest 90 years old.

4.1.3 Patient material in study III

The study material consisted of 324 astrocytomas, of which 242 were diffusely infiltrating astrocytomas (grade II: 28; grade III: 33; grade IV: 181) and 82 were grade I pilocytic astrocytomas. 187 were primary astrocytomas and 55 were recurrences. Of the grade IV astrocytomas, 10 were gliosarcomas, 1 was a giant cell glioblastoma and 170 were ordinary glioblastomas.

4.2 Processing of the study material

The astrocytoma specimens were initially fixed in 4 % phosphate-buffered formaldehyde and then processed into paraffin blocks. On the basis of hematoxylin and eosin-stained slides, a neuropathologist evaluated the tumors according to WHO 2007 criteria (Louis et al., 2007). One histologically representative tumor region was selected from each specimen, and from these areas round punches of 1000 μm in diameter were cut and subsequently collected and mounted into tissue microarray (TMA) blocks. From these blocks, consecutive slices were cut and fixed on

microscope glass slides, which were finally stained immunohistochemically for the studied molecules.

4.3 Immunohistochemistry

For C4d immunostaining, fully automated immunostaining was performed by Ventana BenchMark LT Automated IHC Stainer (The BenchMark Series automated slide preparation system by Ventana Medical Systems, Tucson, AZ, USA). Ventana EZ Prep solution (catalogue No 950–100, Ventana) was used for deparaffinisation. For epitope retrieval CC1: Tris -EDTA buffer pH 8.0 (catalogue No 950–124,Ventana) was used at 95°C to 100°C for 30 minutes. The slides were rinsed between steps with Ventana Tris-based Reaction buffer (catalogue No. 950–300, Ventana). Slides were incubated at 37°C for 32 minutes with a C4d specific rabbit polyclonal anti human C4d antibody (Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria) using a 1:10 dilution. The staining kit used was the Ventana Ultraview DAB Detection Kit. In order to compare the C4d endothelial staining extent with the overall tumor vascularity, a second slide from each TMA block was stained with CD34 antibody. Lyophilised mouse monoclonal antibody, specific to the human CD34 molecule (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) was used as an endothelial cell marker. The dilution used was 1: 500.

R132H point mutation specific mouse monoclonal antibody (Dianova GmbH, Hamburg, Germany) was used to detect *IDH1* gene mutations. Fully automated immunostaining was performed by a Bondmax immunostainer (Leica Biosystems Newcastle Ltd, Newcastle upon Tyne, United Kingdom). Bond Dewax Solution (catalogue No. AR9222) was used for deparaffinisation. For epitope retrieval, RTU Epitope Retrieval Solution 1, pH 5.9-6.1 (catalogue No. AR9961) was used for 30 min at 100°C. The slides were incubated for 30 minutes at room temperature with the *IDH1* antibody (dilution 1:50). The staining kit used was Bond Refine Detection kit. The slides were rinsed between steps with Bond Wash Solution (catalogue No. AR9590).

HIF-1alpha and Hsp27 immunostaining was performed by means of the fully automated Ventana BenchMark LT Automated IHC Stainer (the BenchMark Series automated slide preparation systems by Ventana

Medical Systems, AZ, USA). Ventana EZ Prep solution (catalogue No 950-100) was used for deparaffinisation. For epitope retrieval Tris -EDTA buffer pH 8.0 (catalogue No 950-124) was used at 95°C to 100°C for 30 minutes in the case of the Hsp27 immunostaining and for 90 minutes in the case of the HIF-1alpha immunostaining. Slides were rinsed between steps with Ventana Tris-based Reaction buffer (catalogue No. 950-300). Slides were incubated at 37°C for 32 minutes with specific antibodies. Mouse monoclonal antibody specific for HIF-1alpha (Abcam, Cambridge, UK) was used with a 1:50 dilution. Hsp27 specific antibody (Thermo Fisher Scientific, Fremont, CA, USA) was used with a 1:400 dilution. The staining kit used was the Ventana Ultraview DAB Detection Kit.

For polySia and NCAM, the TMA samples were stained immunohistochemically and confocal microscopy of polySia and NCAM staining was performed. PolySia-binding fluorescent fusion protein (EndoNA2-GFP) was used for polySia detection at a concentration of 10 μg/ml. For NCAM mouse anti-human NCAM antibody (123C3) at a concentration of 4 μg/ml was used as a primary antibody. Immunohistochemical incubations were performed overnight at 4°C. In immunofluorescence, Alexa Fluor 594 chicken anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) were used, and slides were mounted with Immu-Mount (Shandon, USA). For confocal microscopy, a Leica TCS SP MP confocal microscope equipped with a Spectra-Physics Tsunami Ti-sapphire laser and Leica confocal software was used in analysis. Sections were examined at two excitation wavelengths: 488 nm for polySia-binding fusion protein EndoNA2-GFP and 546 nm for fluorescent secondary antibodies.

CA IX immunostaining and evaluation of the staining was performed as described previously (Haapasalo et al., 2006). Also cell proliferation index Ki-67/MIB-1 and p53 immunohistochemistry were performed and analysed as previously described (Sallinen et al., 1994; Haapasalo et al., 1999). EGFR amplification status was examined by means of chromogenic in situ hybridization (CISH), as previously described (Tanner et al., 2000; Järvelä et al., 2006).

4.4 Evaluation of immunohistochemical staining

Extent of C4d immunostaining was examined by area fractions of the stained tumor samples, using a point counting grid. The method has been previously described by Siitonen et al. (1995). In this method, the sample is viewed with a microscope at a magnification value of x400. The grid is placed over the tumor area in order to be subsequently measured and the points covering the area are counted. A value was given to each sample using the formula ((Σ C4d-P)/(Σ Pt)) x 100, where " Σ C4d-P" is the number of C4d positive points in the measured area and " Σ Pt" is the number of all points contained by the grid covering this area. C4d positive tumor area fractions and C4d positive endothelium area fractions were counted separately.

The tumor samples were divided into three groups according to the extent of the staining reaction on the grounds of the C4d positive tumor area fraction. 1 = no positivity, 2 = 2 % or less positive tissue, 3 = more than 2 % positive tissue. The tissue samples were also grouped according to the C4d positive endothelial area fraction: 1 = no positively stained endothelium was seen, 2 = 0.5 % or less of the tissue area was positively stained endothelium, 3 = more than 0.5 % of the tissue area was positively stained endothelium. In addition to the extent thereof, the staining intensity of C4d was also evaluated. The positive samples were considered to be slightly, moderately or strongly positive.

CD34 positive area fraction of the tumor sample was counted using the same morphometric method as for the C4d fractions. CD34 was assumed to stain specifically endothelial cell cytoplasm, thus value acquired from the morphometric count was considered to represent the percentage of total endothelial area of the tumor sample. To compare the extent of endothelial C4d with overall vascularity of the tumor, a variable was created in which the percentage of C4d positive endothelial area fraction was divided by CD34 area fraction percentage.

After the immunohistochemical staining of *IDH1*, Hsp27, HIF-1alpha, polySia and NCAM was performed, the quality, intensity and patterns of staining were studied by means of microscopy. *IDH1* staining was considered either positive or negative, i.e. the tumor was either *IDH1* mutated or not. Hsp27 and HIF-1alpha staining was also evaluated according to the intensity of the staining and the samples were divided

into subgroups on the basis thereof as slight, moderate or strong positivity. For polySia and NCAM, the samples were considered to be simply either positive or negative.

4.5 Statistical analysis

The statistical analyses were performed using IBM SPSS 20.0 software. Significance of associations were defined using Kruskal-Wallis test, Mann-Whitney test and chi-square test. The significance of correlations was determined with Pearson correlation. Kaplan-Meier curves and log-rank test were used in the univariate survival analyses. Cox Regression analysis was used for multivariate survival analyses.

5 RESULTS

5.1 Immunohistochemical results of studies I-III

C4d positive staining was observed in 75 % of grade I-IV astrocytomas studied. C4d positive tumor area fractions were between 0–85 % (mean 6.7 %). C4d positive endothelium area fractions were between 0–4 %, (mean 0.2 %). C4d positive staining was seen in endothelial cell cytoplasm, as well as in astrocytoma cell cytoplasm and tissue extracellular matrix. It was a common occurrence that the tumor cells and the endothelial cells stained strongly within the same area. All of these staining patterns were considered as truly positive staining and were taken into account when the extent and intensity of the staining was evaluated. No other part of tumor tissue than endothelial cells was detected to be CD34 immunopositive.

Similarly to C4d, Hsp27 showed a very heterogeneous staining pattern. Hsp27 immunopositivity was seen in astrocytic tumor cells, in endothelial cells of the tumors as well as in extracellular matrix of tumor tissue. When expression of these molecules (i.e. staining intensity and extent) was evaluated, only cytoplasmic staining was taken into account. This differed from evaluation of C4d staining and was used because it seems to be the most common interpretation method of the staining in previous studies conducted on Hsp27 in CNS. There was a difference in the Hsp27 staining patterns between different grades of astrocytomas. In GBMs, the staining was often more intense than in lower grade astrocytomas. However, the positive staining varied widely even within tumor samples; faintly and moderately stained cells were often seen side by side. Cytoplasmic Hsp27 positive staining was seen in 43.3 % of the diffusely infiltrating astrocytomas studied.

Guidelines for interpretation of immunohistochemical staining of *IDH1* mutation and HIF-1alpha were provided in detail by the manufacturers of the antibodies which were used in the staining procedures. Intense and widely distributed *IDH1* staining in cells was considered as truly positive

staining. HIF-1alpha staining was considered to be truly positive if the staining was located in the tumor cell nucleus. *IDH1* immunopositive infiltrating tumor cells surrounded by normal cells were often detected at the border of the tumor and normal CNS tissue. HIF-1alpha was strongly expressed in tumor cell nuclei in perinecrotic areas. However, HIF-1alpha expression was also found in non-necrotic tumors and in non-necrotic areas of tumor tissue. A proportion of 25 % of the tumor material stained positive for *IDH1* mutation, whereas 79 % stained positive for HIF-1alpha.

PolySia positivity was seen in 19 % of the diffusely infiltrating astrocytomas. 38 % of these tumors were also positive for NCAM. All tumors with polySia were also positive for NCAM, whereas 19 % of the tumors stained positive for NCAM but not for polySia. In pilocytic astrocytomas, only one of the 82 tumors expressed polySia, whereas NCAM positivity was detected in 27 % (n = 22) of these tumors. In all tumor grades the simultaneous expression of polySia and NCAM was localised to cell surfaces concomitantly, which indicated a polysialylated NCAM.

5.2 Clinicopathological correlations

Intensity of C4d staining was found to correlate with the grade in diffusely infiltrating astrocytomas ($p = 0.034$, Kruskal-Wallis test). The C4d positive tumor area fraction also correlated with the grade in diffusely infiltrating astrocytomas ($p = 0.016$, K-W test). Both C4d intensity and extent increased progressively in line with the malignancy of the grade II-IV astrocytoma. When the pilocytic and diffusely infiltrating astrocytomas were compared, it was found that C4d positive tumor area percentages were higher in the pilocytic tumors of grade I than in the grade II-IV diffusely infiltrating astrocytomas ($p = 0.041$, Mann–Whitney test). Figure 3 demonstrates this phenomenon in a box-plot.

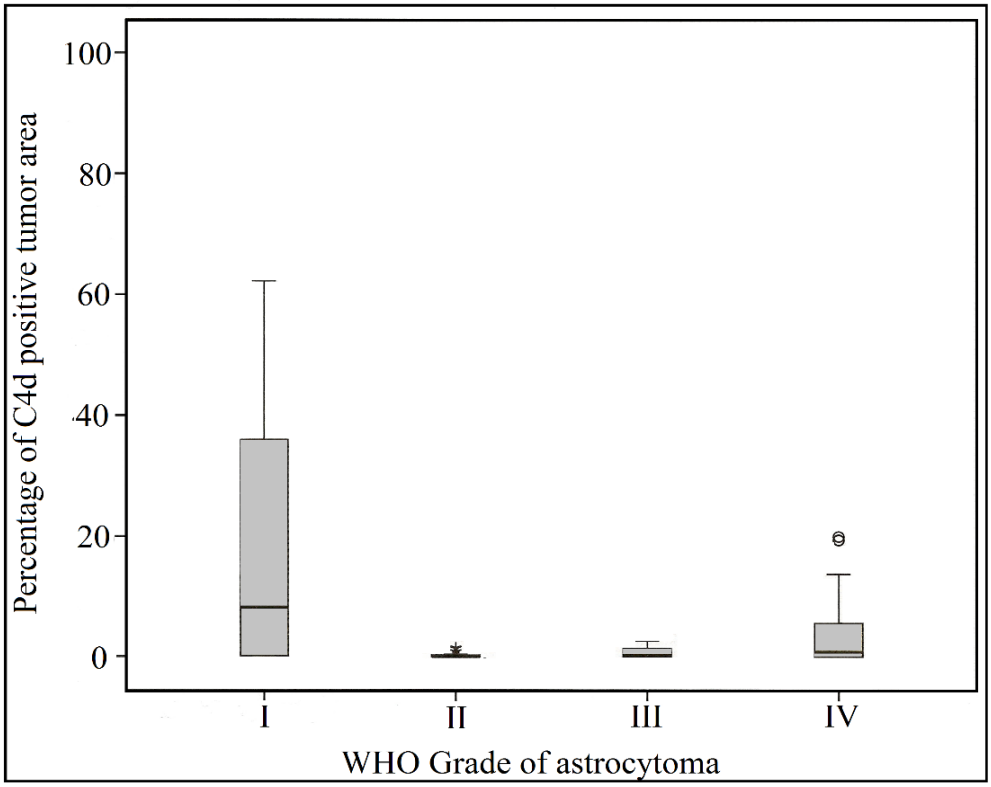

Figure 3. Box-plot of C4d positive tumor area fraction in the WHO grade I-IV astrocytomas.

Furthermore, the Hsp27 and HIF-1alpha expression correlated significantly with increasing grade of grade II-IV astrocytoma (for both $p < 0.001$, chi-square test). In contrast *IDH1* immunostaining positivity was found to correlate with a lower grade of diffusely infiltrating astrocytoma ($p < 0.001$, chi-square test). Of the primary GBMs, 14 out of 196 (7.1 %) exhibited the *IDH1* mutation, whereas in secondary GBMs ($n = 9$), 4 were *IDH1* mutated (44.4 %) ($p < 0.001$, chi-square test). CA IX, polySia or NCAM expression were not associated with the WHO grade.

Table 1 demonstrates the immunohistochemical staining of C4d, *IDH1* mutation, Hsp27, HIF-1alpha, polySia and NCAM in different grades of diffusely infiltrating astrocytomas.

A difference in C4d staining was found between primary and recurrent grade II-IV astrocytomas. Compared to the primary tumors, recurrent tumors had more C4d positive samples when the extent of staining was evaluated. C4d positive area fraction was over 0 % in 46 % of the primary

tumors, whereas it was over 0 % in 65 % of the recurrent tumors (p = 0.047, chi-square test). Comparing the other C4d variables between the primary and recurrent tumors, no statistically significant differences were found. Also in the case of polySia-NCAM, no difference in expression was found between primary vs. recurrent tumors (p = n.s, chi-square test).

The proliferation index Ki-67/MIB-1 was correlated to the molecules studied in study II and III. The tumors with *IDH1* mutation were less proliferative when measured by Ki-67/MIB-1. Median value for *IDH1* mutated tumors was 4.9 % (mean 9.6, SD ± 11.2) and 10.5 % (mean 14.2, SD ± 11.9) for non-mutated tumors (for both p = 0.001, Mann-Whitney test). Also HIF-1alpha correlated with Ki-67/MIB-1 (p = 0.003, Mann-Whitney U-test). Tumors not expressing HIF-1alpha had a median Ki-67/MIB-1 index of 6.8 % and a mean of 11.5 % (SD ± 11.7). In HIF-1alpha positive tumors the median Ki-67/MIB-1 index was 13.2 % and the mean 14.8 % (SD ± 10.9). Hsp27 did not correlate significantly with Ki-67/MIB-1.

A positive polySia expression was also found to correlate with increasing proliferation index (p = 0.007, Kruskall-Wallis test). In study III, p53 expression and EGFR amplification were also studied. PolySia was not associated with p53 expression or with EGFR amplification (p = n.s., chi-square test).

	Grade II	Grade III	Grade IV	Total	p-value
C4d	52.4 %	75.0 %	80.4 %	74.5 %	0.034
IDH1	73.3 %	69.4 %	11.6 %	25.0 %	<0.001
Hsp27	16.3 %	32.3 %	50.2 %	43.4 %	<0.001
HIF-1alpha	10.5 %	20.0 %	43.5 %	79.3 %	<0.001
polySia	7.1 %	24.2 %	19.3 %	18.6 %	n.s.
NCAM	32.2 %	54.5 %	35.9 %	38.0 %	n.s.

Table 1. Percentages of the positively stained (immunohistochemistry) samples for C4d, *IDH1* mutation, Hsp27, HIF-1alpha, polySia and NCAM in diffusely infiltrating astrocytomas. In this table, C4d means the intensity of staining. The percentages in the extent area fraction are presented in the original communication.

5.3 Correlations between the studied molecules

First of all, a significant correlation between the CD34 endothelial area fraction and C4d endothelial area fraction was found in diffusely infiltrating astrocytomas ($r = 0.381$, $p < 0.001$, Pearson Correlation). The proportion of C4d positive endothelium to overall vascularity (C4d / CD34) exhibited an increasing trend in line with the tumor grade, but no significance was achieved in the statistical analysis. C4d did not correlate significantly with *IDH1* mutation, nor with Hsp27, HIF-1alpha or CA IX, although there was once more a trend indicating that Hsp27 expression and C4d expression would rise simultaneously.

HIF-1alpha, Hsp27 and CA IX were studied for their correlations with *IDH1* mutation status and also for their correlations with each other. In the entire cohort, a significant correlation was found between HIF-1alpha and Hsp27 ($p = 0.020$, chi-square test). HIF-1alpha was also found to correlate with CA IX in the entire cohort ($p = 0.005$, chi-square test). No significant association was found between Hsp27 and CA IX (p = n.s., chi-square test).

In the entire cohort, a significant correlation between *IDH1* mutation and HIF-1alpha was found ($p = 0.001$, chi-square test). Positive staining for both the *IDH1* mutation and HIF-1alpha was detected simultaneously. There was also a correlation between *IDH1* mutation and CA IX immunopositivity ($p = 0.029$, chi-square test). An association was found between Hsp27 positive staining and negative *IDH1* mutation status ($p < 0.001$, chi-square test) both in the entire cohort as well as when studied separately in grade II and III astrocytomas ($p = 0.006$ and 0.002, respectively, chi-square test). Within GBMs, there was no significant correlation between *IDH1* mutation and Hsp27. Regarding grade II-III astrocytomas 9.8 % of *IDH1* mutated tumors showed Hsp27 expression, whereas 55.0 % of *IDH1* non-mutated tumors showed Hsp27 expression. 83.6 % of Hsp27 negative tumors were *IDH1* positive. The Hsp27 expression detected in the *IDH1* mutated tumors was slight in each case; none showed a moderate or a strong expression.

Interesting results were found when the correlations presented ahead were tested again, this time separately in two subgroups, dividing the entire cohort into *IDH1* mutated and *IDH1* non-mutated tumors. When *IDH1* mutated tumors were considered, HIF-1alpha expression

significantly correlated with CA IX positivity (p = 0.027 chi-square test), yet no association was found in *IDH1* non-mutated tumors. In *IDH1* mutated tumors, all HIF-1alpha positive samples expressed CA IX. Hsp27 associated with HIF-1alpha in the entire cohort, as previously presented, however Hsp27 did not correlate significantly with HIF-1alpha expression in *IDH1* mutated tumors.

The expressions of polySia and NCAM also differed depending on the *IDH1* mutation status. Tumors having an *IDH1* mutation were more often polySia-NCAM positive than negative (p = 0.001, chi-square test). Similarly, NCAM positive tumors were more often *IDH1* mutated than non-mutated (p = 0.001, chi-square test).

5.4 Patient survival

5.4.1 Univariate survival analysis

In the univariate survival analysis new information was found on how C4d and Hsp27 expression affects patient prognosis in diffusely infiltrating astrocytomas. In a fiveyear follow-up, increasing intensity of C4d staining was associated with a worsened outcome among patients (p = 0.014, log-rank test). Here the mean lifetime after the primary resection of the tumor was 32.5 months when the intensity was evaluated as negative (95 % confidence interval 21.9-43.0); when the intensity was evaluated as slight the mean lifetime was 29.8 months (CI 21.2-38.3); when the intensity was moderate the mean lifetime was 14.1 months (CI 6.7-21.4) and with a strong intensity of C4d staining, the mean lifetime was 9.7 months (CI 3.0-16.3). The C4d staining extent did not correlate significantly with survival.

Also increasing Hsp27 expression predicted worse rates of patient survival in the univariate survival analysis than if the tumor was Hsp27 negative (p = 0.001, log-rank test). A correlation between the HIF-1alpha expression and a patient's worsened outcome was also found (p = 0.001, log-rank test). CA IX expression correlated significantly with shorter patient survival (p = 0.022). No significant associations were found when the univariate survival studies were conducted separately in different grades concerning C4d, Hsp27 and HIF-1alpha. However, in the case of *IDH1* mutation, when the survival analyses were performed grade by

grade, a significant prognosis difference was found only in GBMs, in which *IDH1* mutation was associated with better patient outcome ($p < 0.001$). Also in the entire cohort, *IDH1* mutation positivity was found to be associated with longer patient survival ($p < 0.001$, log-rank test). The Kaplan-Meier survival curves drawn in the univariate survival analyses of study II are shown in Figure 4.

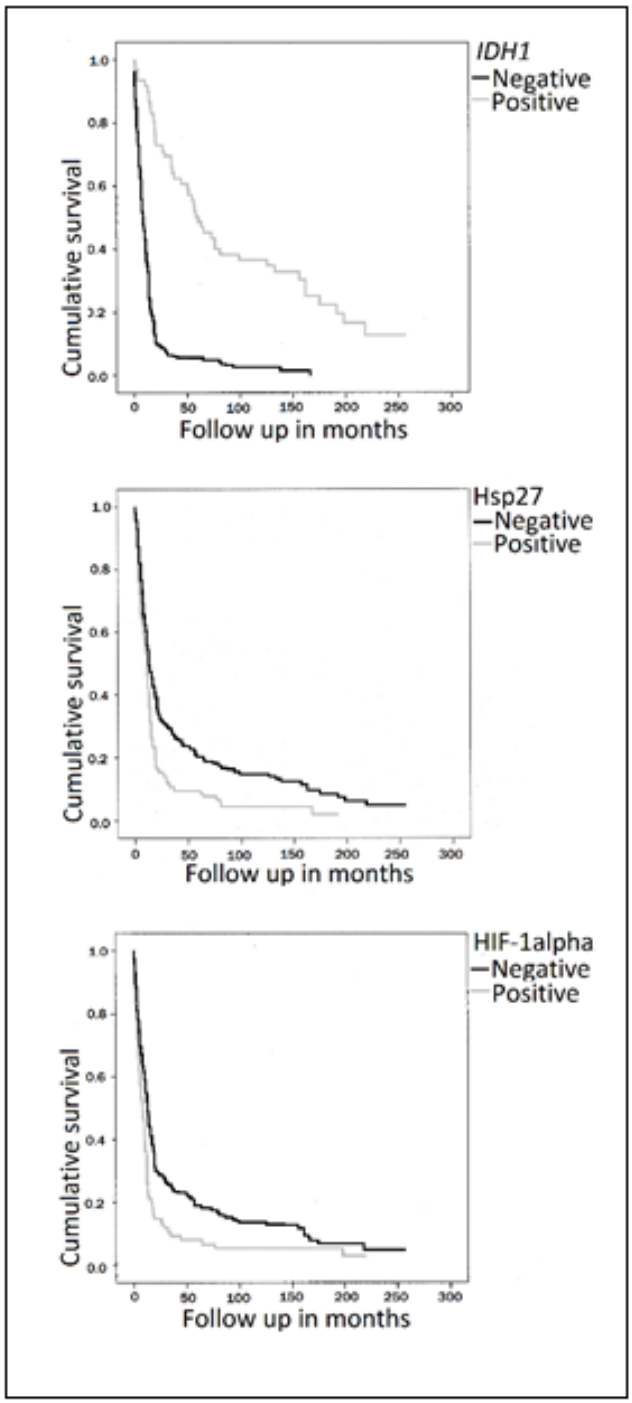

Figure 4. Association of Hsp27, HIF-1alpha and the *IDH1* mutation to the patient prognosis. Kaplan-Meier survival curves of WHO grade II-IV diffusely infiltrating astrocytomas (p = 0.001).

PolySia positivity was associated with better patient prognosis within the entire cohort (p = 0.020, log-rank test), likewise NCAM (p= 0.035, loq-rank test). In both instances, no association was found when studied separately in grades II and III. However, in glioblastomas, a positive polySia expression was significantly associated with a better prognosis (p = 0.005, log-rank test).

In further analyses involving only glioblastomas, both the polySia and NCAM expressions were assessed simultaneously. Patients with both polySia and NCAM expression had longer survival rates than the patients with negative polySia and positive or negative NCAM (p = 0.014, log-rank test, Figure 5). In addition, patients with NCAM expressing tumor had the worst prognosis compared to the patients whose tumor was NCAM negative or expressed both NCAM and polySia.

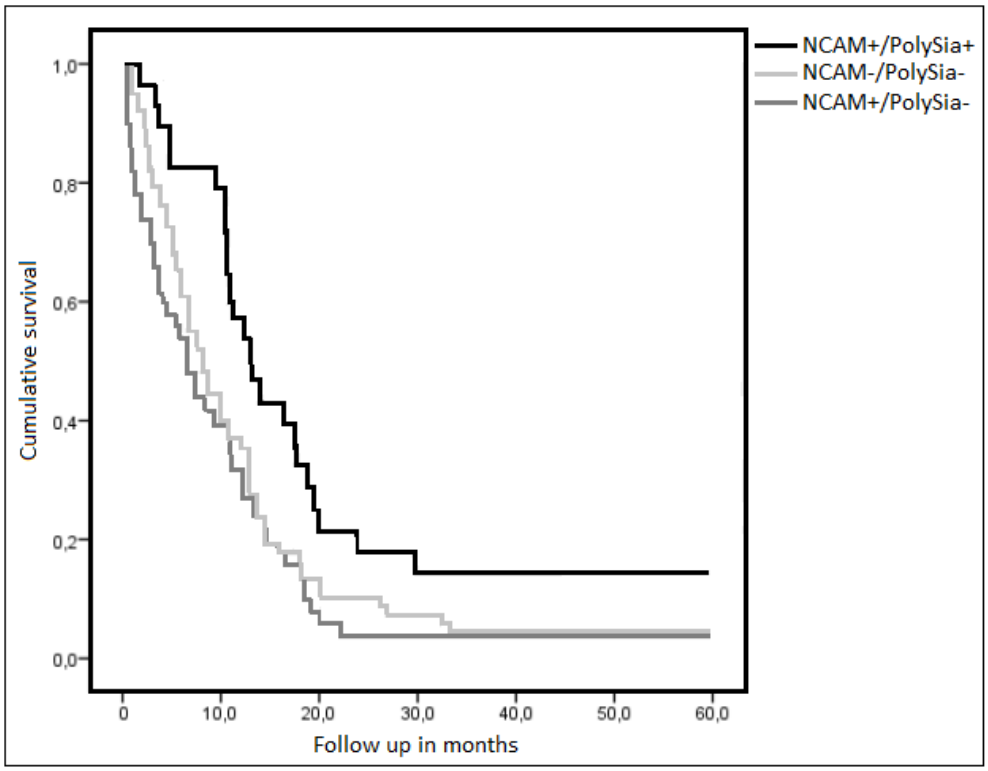

Figure 5. Kaplan-Meier curves for glioblastoma patient survival. The figure demonstrates how patients with GBM expressing polysialylated NCAM have a better prognosis than patients with GBM expressing solely NCAM or if the GBM is negative for both polySia and NCAM (p = 0.014, log-rank test).

5.4.2 Multivariate survival analysis

Cox's stepwise regression model was used in all the multivariate survival analyses performed. The multivariate survival analyses were performed in all studies I-III. In study I, only the WHO grade came up as an independent prognostic factor (Hazard ratio = 5.666, 95 % confidence interval 3.069 – 10.459). Other parameters included within the scope of the analysis were the C4d intensity, C4d extent and patient age. Consequently, C4d is not an independent prognosticator.

In study II, the following parameters were included into the multivariate survival analysis: expression of Hsp27, HIF-1alpha and CA IX, *IDH1* mutation, WHO grade, whether chemotherapy had been received (yes or no), radiotherapy received (yes or no), and patient age. Patient age, *IDH1* mutation and HIF-1alpha appeared as independent prognostic factors. The hazard ratio (Exp(B)) for the patient age was 2.299 (95 % CI for Exp(B) 1.880-2.840). Exp(B) for *IDH1* was 0.206 (95 % CI for Exp(B) 0.126-0.337). Exp(B) for HIF-1alpha was 1.579 (95 % CI for Exp(B) 1.111-2.245). The patient age cut off points were 57 and 72 years. A hazard ratio below 1 indicates a favourable prognosis whereas a hazard ratio above 1 indicates an unfavourable prognosis.

In study III the multivariate survival analysis was conducted with the following variables: patient age, *IDH1* mutation, EGFR amplification, Ki-67/MIB-1 labelling index, polySia expression, NCAM expression and WHO grade. *IDH1* mutation, patient age, Ki-67/MIB-1 and polySia expression came up as independent prognostic factors in this order in the stepwise Cox model. The hazard ratio (Exp(B)) for *IDH1* was 0.195 (95 CI for Exp(B) 0.101-0.376). Exp(B) for patient age was 1.705 (95 % CI for Exp(B) 1.217-2.390). Exp(B) for Ki-67/MIB-1 was 1.673 (95 % CI for Exp(B) 1.210-2.312). Exp(B) for polySia was 0.516 (95 % CI for Exp(B) was 0.280-0.951). Thus *IDH1* mutation was found to be an even stronger prognosticator than the patient age. The age cut off points were 54 and 70 years. The cut off points for Ki-67/MIB-1 were 5 % and 15 %.

6 DISCUSSION

The acquisition of the core cancer hallmarks is enabled by the impact of the tumor microenvironment, in which hypoxia and inflammation are greatly involved. Both cause changes in the gene expression of the neoplastic cells and tumor microenvironment cell recruitment, which in turn leads to malignant progression of the tumor. The malignant progression includes the epithelial-mesenchymal transition, leading to cell migration and tumor invasiveness. Additionally, angiogenesis and resistance to chemotherapy and radiation therapy are functions enforced by the actions of the tumor microenvironment. In practice, function of the tumor microenvironment can be seen in expression of specific molecules. Inflammation is marked by complement activation and expression of C4d. The HIF-1alpha transcription factor is upregulated under hypoxic conditions, leading to upregulation of Hsp27 and CA IX. Polysialylation of NCAM is associated to tumor malignancy and suggested to loosen cell adhesions.

Expression of C4d and Hsp27 and their relation to the clinicopathological features of astrocytomas had not been comprehensively assessed before. Additionally, the association of polySia and NCAM to astrocytoma patient survival is yet to be comprehensively studied. *IDH1* mutation is a remarkable feature of WHO grade II and III astrocytomas, as well as secondary GBMs. *IDH1* mutation alters the cell gene expression, yet not all the proteins under the impact thereof have been found. Furthermore, the point in the pathway affected by the mutation is unknown in many cases.

6.1 Study I – Expression of C4d in astrocytomas

In study I C4d was found to correlate with the tumor grade in astrocytomas. In this study, pilocytic astrocytomas were also included in the analysis. Interestingly pilocytic astrocytomas showed more intense

and extensive staining of C4d than the diffusely infiltrating astrocytomas. Within the diffusely infiltrating astrocytomas the C4d appearance increased in line with the tumor malignancy. However, even the GBMs did not stain as extensively and intensively as the pilocytic astrocytomas.

A difference was also seen between the recurrent and primary resection of the tumor. Recurrent astrocytomas were more often C4d positive than the primary tumors. This is probably due to prolonged inflammation at the peritumoral area or reactive inflammation enhanced by surgery-provoked injuries (Liu el al., 2008; Hamard et al., 2016). Hamard et al. suggest that the surgical brain injury provokes inflammation at the surgical site, thus enhancing the recurrence and progression of malignancy. They also suggest that manipulation of the inflammatory response after surgery could prevent local recurrence and enhance effectiveness of other therapies.

Why benign pilocytic astrocytomas stain more extensively C4d positive than the malignant diffusely infiltrating astrocytomas is probably due to the complement inhibitory factors expressed in the malignant astrocytomas. These include complement regulatory proteins (CRPs) and complement inhibitors factor H and factor H-like proteins (Shinoura et al., 1994; Mäenpää et al., 1996; Junnikkala et al., 2000). Mäenpää et al. suggest that malignant glioma cell lines are highly resistant to complement mediated immune destruction because of the widespread expression of CRPs on their cell membrane. Complement inhibitors factor H and factor H-like proteins have been shown to be involved in resistance of complement-mediated killing in GBMs. Thus, the complement cascade activation is reduced in diffusely infiltrating astrocytomas compared to benign pilocytic astrocytomas, probably explaining why the malignant tumors stain C4d positive to a lesser extent than the pilocytic astrocytomas. Furthermore, pilocytic astrocytomas and GBMs both show a glomeruloid vascular proliferation. It has been shown that the complement cascade activates more easily in stressed endothelial cells than in the cells under normal physiological conditions (Yin et al., 2007). Thus, the pathological blood flow may promote complement activation. This may also explain how pilocytic astrocytomas and GBMs extensively exhibit a C4d staining. C4d did not correlate with *IDH1* mutation status. However, when the staining was evaluated both extracellular and intracellular C4d staining was taken into account. Complement

components may be produced locally, or they may relocate to the tumor stroma also from plasma through the damaged blood brain barrier (Shastri et al., 2013). Thus there may be an extracellular C4d expression in the tumor regardless of the *IDH1* mutation status.

The proportion of C4d positive endothelium to overall vascularity had a rising trend along with the tumor grade, but there were no statistical significance. This means that in vessels of more malignant tumors the complement cascade could activate more often than in vessels of lesser grade tumors. This was expected, since the complement activates more easily in stressed endothelial cells than in those under normal conditions (Yin et al., 2007). This could particularly reflect the vasculature of GBMs, which has tendency to complex glomeruloid formation. However, the fact that pilocytic astrocytomas share this trait could confuse the statistical analyses.

6.2 Study II – Hypoxia biomarkers, their interrelations and impact of *IDH1* mutation on their expression

In study II the hypoxia related molecules HIF-1alpha and Hsp27 correlated to tumor grade. It seems that the expression of these molecules is upregulated as the malignancy of the tumor increases. However, *IDH1* mutation was associated with a lower grade, in line with previously published literature. In the entire cohort, HIF-1alpha was significantly associated with both Hsp27 and CA IX expression, as well as with the *IDH1* positive mutation status. This is not a surprising result either and complies with previous studies. CA IX expression associated significantly with positive *IDH1* mutation status. However, Hsp27 expression was significantly scarcer in *IDH1* mutated tumors than in *IDH1* non-mutated tumors. Also Hsp27 expression detected in the *IDH1* mutated tumors was slight in each case; no case showed a moderate or a strong expression.

The *IDH1* mutation correlated significantly with HIF-1alpha expression. The HIF-1alpha expression also seemed to rise in line with increasing tumor grade. With reference thereto, it should be borne in mind that hypoxia is the greatest factor to upregulate the HIF-1alpha expression (Fábián et al., 2016). Moreover, in the study material of study II, HIF-1alpha expression was strong in the perinecrotic areas, indicating

that hypoxia was present. Thus, in glioblastomas, in which severe hypoxia occurs and the *IDH1* mutation is usually absent, HIF-1alpha expression is most likely upregulated via hypoxia related pathways. However, as hypoxia is not that frequent in lower grade astrocytomas, it could be speculated that the *IDH1* mutation, rather than hypoxia, is involved in HIF-1alpha upregulation in these tumors. Enzymes PHD and FIH are inactive in *IDH1* mutant cells due to the depletion of alpha-KG. Normally, both limit HIF-1alpha expression. Thus, a loss of their function in the *IDH1* mutant cells can lead to accumulation of HIF-1alpha. In this manner, the HIF-1alpha expression could also be upregulated by a hypoxia independent mechanism.

The HIF-1alpha expression correlation to Hsp27 and CA IX expression changed when the entire cohort was divided by the *IDH1* mutation status. Understandably, HIF-1alpha did not correlate with Hsp27 in the *IDH1* mutated tumors, since Hsp27 expression in these tumors was scarce. However, in the *IDH1* mutated tumors, all samples expressing HIF-1alpha showed CA IX expression. In *IDH1* negative tumors significant correlation between HIF-1alpha and CA IX expression was lost.

Thus, it seems that *IDH1* mutation is involved in the expression pathways of Hsp27 and CA IX. Certainly, it has been previously known that expression of Hsp27 can also be regulated by other mechanisms and transcription factors other than hypoxia and the HIF-1alpha pathway (de Thonel et al., 2012). Since expression of Hsp27 was scarce in *IDH1* mutated tumors, it could be speculated that *IDH1* mutation has an effect on the Hsp27 expression pathway to some extent. As indicated previously, the *IDH1* mutation can change the cell's protein expression at least by means of the inhibition of the alpha-KG dependent dioxygenases or by altering DNA and histone methylation. It has been previously shown that *IDH1* mutation increases expression of HIF-1alpha, as formerly demonstrated in the results of study II in this thesis. Since HIF-1alpha is a direct transcription factor of Hsp27, it could be speculated that solely the inhibition of alpha-KG dependent enzymes would not be sufficient in order to prevent the Hsp27 expression. This way, the pathway of Hsp27 expression is probably down regulated by direct disturbance of the Hsp27 gene transcription. Furthermore, supporting this hypothesis, it can be moreover speculated that in these tumors, Hsp27 could also be upregulated by other mechanisms than the hypoxia related HIF-1alpha

pathway. These other mechanisms include for example oxidative stress. Production of 2-HG by the mutated *IDH1* enzyme consumes cell's NADPH reserves and exposes the cell to reactive oxygen species damage (Liu and Ling, 2015). For example, NADPH is essential to glutathione and thioredoxin when regulating intracellular redox state. Hsp27 is upregulated in cells under oxidative stress, thus it could be presumed that Hsp27 would be upregulated in *IDH1* mutated cells because of ineffective reactive oxygen species elimination. This also leads to suggest that the entire Hsp27 gene transcription is disturbed in *IDH1* mutated tumors.

The Hsp27 association with HIF-1alpha in the entire cohort could be explained by the fact that the majority of the study material (74.9 %) consisted of GBMs. In these tumors *IDH1* mutation is rather rare and hypoxia is substantial, amongst other Hsp27 expression enhancing factors.

In *IDH1* mutated tumors HIF-1alpha and CA IX expression was concomitant; all tumors expressing HIF-1alpha also expressed CA IX. No significant correlation was found when the correlation was studied in *IDH1* non-mutated tumors. A loss of FIH1 and PHD regulation in the *IDH1* mutated cells could lead to a freely flowing HIF-1alpha-CA IX expression pathway, causing CA IX overexpression. Nevertheless, it is curious why HIF-1alpha and CA IX did not correlate in *IDH1* non-mutated tumors, of which the majority are hypoxic GBMs. The expression of CA IX seems to be able to also initiate hypoxia independently (Ihnatko et al., 2006; Kallio et al., 2010), however the HIF-1alpha pathway seems to be the main regulator of the expression thereof and it has been questioned whether the CA IX expression without HIF-1alpha is even possible (Wykoff et al., 2000; Kaluz et al., 2009). Kaluz et al. state in their review that HIF-1alpha expression is essential for CA9 transcription, yet they do disclose that hypoxia independent expression of CA IX is possible (Kaluz et al., 2009). It could then be presumed that HIF-1alpha and CA IX expression would correlate in the hypoxic GBMs, in which hypoxia is a core element, which however was not the case. Thus, it seems that in diffusely infiltrating astrocytomas that are *IDH1* non-mutated, the hypoxia independent expression of CA IX could also occur and it can be questioned whether this could occur independently from HIF-1alpha.

On the other hand, the fact that the majority of the tumor material consisted of GBMs is demonstrated in the correlation found between HIF-

1alpha and the increasing cell proliferation index. In GBMs, the cell proliferation is high and the HIF-1alpha expression is elevated under hypoxic conditions, thus they are apparent concomitantly.

The impact of the *IDH1* mutation on hypoxia biomarkers has been previously studied with the results differing from those presented in this thesis (Metellus et al., 2011). Metellus et al. did not find a significant correlation between the *IDH1* mutation status and HIF-1alpha or CA IX. The study material consisted of 33 WHO grade II-III gliomas, including only four astrocytomas. The rest of the study material consisted of 13 oligoastrocytomas and 16 oligodendrogliomas. Thus, the study material was scarce regarding astrocytomas and the results mainly represent oligodendrocytic tumors.

6.3 Study III – Expression of polySia and NCAM in astrocytomas

In study III no association between the WHO grade of astrocytoma and polySia or NCAM was found. As presented in the literature review, polySia has been previously connected with an increasing tumor malignancy, whereas NCAM expression has been shown to correlate with a lower tumor grade. However, both have been connected to tumor invasiveness.

The survival analysis of study III provided new information on how the expression of polySia and NCAM effects on the patient prognosis. PolySia expression was associated with a better prognosis when all diffusely infiltrating astrocytomas were included in the analysis, but also when the GBMs were studied separately. NCAM expression was also associated with longer patient survival in the diffusely infiltrating astrocytomas, but no association was found when different tumor grades were studied separately. In GBMs, patients having tumors with both polySia and NCAM expressions had a longer survival time than the patients with negative polySia and positive or negative NCAM. Additionally, patients with NCAM expressing tumors had the worst prognosis compared to patients whose tumor was NCAM negative or expressed both NCAM and polySia. Thus, it seems that in GBMs, polysialylation of NCAM is favourable in terms of patient prognosis.

PolySia has been previously associated with tumor malignancy, thus it could be presumed that the patient prognosis would also be worsened

when tumor expresses polySia. However, it could be stated that similarly to the *IDH1* mutation, polySia expression is a feature of malignant tumors, but within them the latter is associated with a favourable patient prognosis. The specific mechanisms of how they affect on the prognosis remain unknown in both cases. On the other hand, a strong association of polySia to other outcome improving features, such as the association to the *IDH1* mutation, could have a strong enough effect in the survival analysis, also showing polySia as a favourable feature. However, the fact that the polySia expression maintained the association to a favourable outcome when studied within GBMs, indicates that the results are independent from the impact of the *IDH1* mutation, since *IDH1* mutation is rare in primary GBMs, which concluded the main proportion of the GBMs in the study material. Moreover, in the multivariate survival analysis polySia came up as an independent prognosticator, indicating a favourable patient outcome.

Only one of 82 pilocytic astrocytomas expressed polySia, whereas 22 tumors expressed NCAM. This is in line with previous findings showing that NCAM is a feature associated with lower tumor grades, whereas polySia associates with higher grades. The survival analysis performed with GBM patients only indicates that NCAM in higher grade tumors may impact patient prognosis unfavourably, at least compared to polysialylated NCAM. Although in GBMs no direct association between a worsened outcome and NCAM expression was seen, compared to the polySia expression, it was indicated that the patient prognosis was worse in NCAM expressing tumors than in the NCAM non-expressing tumors. Yet again, as previous studies indicate, NCAM is more likely to be a feature of benign than malignant tumors, but when expressed in higher grade tumors it could affect patient prognosis unfavourably.

Upon comparing the results of study III to previously published studies on astrocytomas (Petridis et al, 2009; Amoureux et al., 2010), it could be stated that in study III the study material and results benefit from a larger patient population, had a longer follow up time, and also included the *IDH1* analysis. This probably improves the value of study III. Furthermore, in previous studies in the survival analysis only polySia-NCAM had been studied, the patient outcome has not been compared between polySia-NCAM and mere NCAM expression. In the study of Petridis et al., showing an association between polySia and increasing

tumor grade, the statistical analysis was performed combining grade I and II astrocytomas as one group and grade III and IV as another. Grade I pilocytic astrocytomas should preferably be studied separately from grade II-IV diffusely infiltrating astrocytomas, since the biological background of pilocytic astrocytomas differs from grade II-IV tumors. Comparing this to the fact that in study III only one pilocytic astrocytoma was polySia positive, the findings of Petridis et al. seem biased due to the grouping of the grades.

In the study of Korja et al., similar results on neuroblastomas have been found than in study III on astrocytomas (Korja et al., 2009). Korja et al. showed that in neuroblastomas the absence of polysialylated NCAM is an unfavourable prognostic marker for advanced stage neuroblastoma. Also in study III, a similar association was found in GBMs, which are also highly malignant tumors. Further speculating, the positive effects of NCAM binding have been previously seen in astrocytomas as well. Similarly to polySia, human natural killer-1 (HNK-1) is a molecule that binds to NCAM and appears on the cell surfaces. In the study of Suzuki-Anekoji et al., HNK-1 expression in astrocytomas was associated with a better patient prognosis, compared to HNK-1 negative cases (Suzuki-Anekoji et al., 2011).

Yet again it seems that *IDH1* mutation status has an effect on expression of both polySia and NCAM. In *IDH1* mutated astrocytomas polySia-NCAM and NCAM expression was significantly more frequent than in *IDH1* non-mutated tumors.

6.4 The research results in practice

In planning new treatment of astrocytic tumors, inflammation and hypoxia related molecules should be taken into account because hypoxia and inflammation have been shown to cause a devastating progression of cancer malignancy affecting on patient prognosis. Inflammation and hypoxia are also involved in the core problem of diffusely infiltrating astrocytomas; the invasion capability. Furthermore, EMT is a result of hypoxia and inflammation that is practically the process of neoplastic cells gaining invasive capabilities. If the processes leading to the EMT could be suppressed by new drugs, it would most likely favour patient prognosis.

HIF-1alpha targeting treatment of cancer has constituted a point of interest lately (Masoud and Li, 2015; Patel and Sant, 2016; Wigerup et al., 2016). This is because HIF-1alpha mediates the expression of many oncogenic and EMT leading proteins in cancer, including Hsp27 and CA IX. Furthermore, the results of study II show that HIF-1alpha is an independent prognostic factor, indicating a worsened patient outcome. However, as presented in this thesis, the HIF-1alpha mediated pathways of protein expression are most likely affected by *IDH1* mutation. If expression of CA IX is targeted, HIF-1alpha inhibition may be insufficient in *IDH1* non-mutated astrocytomas, the HIF-1alpha expression and CA IX expression did not correlate in the case thereof. However, HIF-1alpha has many other target genes that also contribute to the malignant progression (Patel and Sant, 2016). Therefore, conducting research on the HIF-1alpha targeting therapy is valuable, although it would be beneficial to study whether *IDH1* mutation affects their HIF-1alpha mediated expression in astrocytomas.

Of the hypoxia related molecules, Hsp27 has also been shown to be a potential target molecule in the therapy against astrocytomas. Li et al. showed that the Hsp27 inhibitor quercetin causes cell death by blocking autophagy in the GBM cells in vitro (Li et al., 2016). Autophagy is a survival method for cancer cells under hypoxia (Bo et al., 2015). The expression of Hsp27 leads to resistance to t-AUCB, which induces autophagy in the GBM cells (Li et al., 2015; Li et al., 2016b). Additionally, Jakubowicz-Gil et al. showed that blocking of Hsp27 by temozolomide or quercetin leads GBM cells more easily to initiation of apoptosis. A further development of Hsp27 targeting therapy is relevant, as expression of Hsp27 is associated with shorter patient survival.

Besides the invasion capability, it seems that inflammation is another important cancer hallmark to target in therapy. There are two potential mechanisms of the immune system to target; either by inhibiting the harmful pro tumorigenic inflammation or by enhancing the antitumor immune response. Hamard et al. propose that after resection of a brain tumor, surgical brain injury is associated with the tumor promoting inflammation at the surgical site, thus enhancing the recurrence of the tumor (Hamard et al., 2016). Another system to target could be the PD-1/PD-L1 pathway. As formerly presented, the overexpression of PD-1/PD-L1 causes an adverse activation of the immune system in the tumor

microenvironment. The system shifts function of the inflammatory system towards immune evasion. Nivolumab and pembrolizumab are inhibitors for PD-1/PD-L1. They function against cancer by enhancing antitumor immune response. Downregulation of Treg also prevents the pro tumorigenic inflammation. Nivolumab and pembrolizuma in treatment of non-small cell lung cancer have been approved in the United States and have yielded encouraging results (Borghaei et al., 2015). If the tumor expresses C4d in immunohistochemical staining, it is predictable that tumor associated inflammatory cells are present in the tumor stroma. Thus, the TAM and Treg associated anti-inflammatory treatment in the C4d positive tumors could be relevant in order to target recurrence and malignant progression.

However, in order to proceed in finding new treatment methods against astrocytomas, it is important to survey the complete expression pathways of the molecules presented in this thesis. The results obtained from studying the protein expression by immunohistochemistry are directional. Completely reliable results on the protein expression pathways require a DNA specific analysis. Thus it can be suggested that in future research these preliminary results are taken into account and give research topics to study groups specialized studying cell model signalling.

Another topic criticised in the peer review process of the publications presented in this thesis is the usage of the tumor microarray (TMA) samples. They were thought to present a rather regional expression of proteins than the histology of the complete tumor. In all studies, the TMAs were built from hand selected parts of the primary tumor samples. The regions of the samples from which the TMAs were built were evaluated to be histologically representative areas, following the guidelines of the WHO classification of tumors. A concordance between the TMAs and the whole paraffin sections in immunohistochemical studies has been demonstrated repeatedly and in multiple tumor types (Jawhar, 2009).

6.5 Future prospects

Prospectively, the impact of the *IDH1* mutation on polySia and NCAM expression should be further examined, as the possible affected expression pathways remain unclear. Additionally, the manner in which

IDH1 mutation affects the expression of Hsp27 and CA IX on a molecular basis remains unknown and can only be speculated.

Also another interesting, yet rather optional study topic would be to examine the extracellular expression of Hsp27 in astrocytomas. Calderwood and Cong suggest that the heat shock proteins expressed extracellularly in the neoplastic tissues are of growing importance in the etiology of cancer, and may impact powerfully on the tumor immunity and metastasis (Calderwood and Gong, 2016). In study II, the cytoplasmic immunohistochemical staining of Hsp27 was studied. However, the staining of ECM was also seen. Thus it would also be interesting to examine the ECM staining of the Hsp27 of the study material. Also regarding polySia, it would be interesting to study the expression of polySia and NCAM in the specific cellular subpopulations in astrocytomas, since polySia-NCAM has been proposed to be a stem cell marker (Pennartz et al., 2004).

7 SUMMARY AND CONCLUSIONS

The expression patterns of C4d, Hsp27, polySia and NCAM in astrocytomas were assessed in the studies presented in this thesis. New information pertaining to their association to the clinicopathological features was found and the correlation of expression to patient survival was explicated. The expression of C4d and Hsp27 was associated with increasing tumor malignancy and a worsened patient outcome. The polySia and NCAM expression was instead associated with a better patient outcome than if the tumor was polySia or NCAM negative.

IDH1 mutation seemed to have an influence on the expression of Hsp27, CA IX, polySia and NCAM. It can be concluded that presumably Hsp27 expression is blocked at the level of DNA transcription in the *IDH1* mutated tumors. In the *IDH1* non-mutated tumors, CA IX expression seems to also be induced via hypoxia non-related pathways.

If HIF-1aplha mediated expression of CA IX and Hsp27 is targeted in therapy by HIF-1alpha inhibition, acknowledgement of the *IDH1* mutation status is important, since HIF-1alpha pathway of expression of CA IX and Hsp27 seems to be dependent on the *IDH1* mutation status of the tumor. Combining the results of this thesis and of the previous literatures, it could be suggested that in the treatment of the C4d expressing tumors, anti-inflammatory or antitumor immune response enhancing therapy could be relevant.

Expression of C4d and Hsp27 is related to tumor malignancy and may provide guidance in terms of tumor grading, favouring diagnosis of a higher grade tumor. Whereas polySia positivity in immunohistochemistry, most likely rules out diagnosis of pilocytic astrocytoma.

Regarding diffusely infiltrating astrocytomas, invasiveness is a core problem affecting patient prognosis. Astrocytoma targeting therapy would greatly benefit from therapy methods restricting the invasion capability of the tumor. More precisely, targeting the hypoxia and inflammation related molecular mechanisms may lead to recovery of a potent form of therapy.

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