Importance of GFAP isoform-specific analyses in astrocytoma

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Abstract
Gliomas are a heterogenous group of malignant primary brain tumors that arise from glia cells or their progenitors and rely on accurate diagnosis for prognosis and treatment strategies. Although recent developments in the molecular biology of glioma have improved diagnosis, classical histological methods and biomarkers are still being used. The glial fibrillary acidic protein (GFAP) is a classical marker of astrocytoma, both in clinical and experimental settings. GFAP is used to determine glial differentiation, which is associated with a less malignant tumor. However, since GFAP is not only expressed by mature astrocytes but also by radial glia during development and neural stem cells in the adult brain, we hypothesized that GFAP expression in astrocytoma might not be a direct indication of glial differentiation and a less malignant phenotype. Therefore, we here review all existing literature from 1972 up to 2018 on GFAP expression in astrocytoma patient material to revisit GFAP as a marker of lower grade, more differentiated astrocytoma. We conclude that GFAP is heterogeneously expressed in astrocytoma, which most likely masks a consistent correlation of GFAP expression to astrocytoma malignancy grade. The GFAP positive cell population contains cells with differences in morphology, function, and differentiation state showing that GFAP is not merely a marker of less malignant and more differentiated astrocytoma. We suggest that discriminating between the GFAP isoforms GFAPδ and GFAPα will improve the accuracy of assessing the differentiation state of astrocytoma in clinical and experimental settings and will benefit glioma classification.

KEYWORDS
astrocytoma, biomarker, GFAP, GFAP variants, GFAPδ, glioma, intermediate filaments

1 INTRODUCTION
Gliomas are the most common primary tumors of the central nervous system (CNS). Classically, histological assessment has been used to determine glioma subtype and malignancy grade, which is essential for prognosis and treatment strategies (Wesseling, Kros, & Jeuken, 2011). Since 2016, the World Health Organization (WHO) classification system is applying additional molecular information to improve CNS tumor diagnostics (Louis et al., 2016). This system distinguishes different subtypes of low- and of high-grade glioma based on mutations in isocitrate dehydrogenase 1 (IDH1), and codeletion of the short arm of chromosome 1 and the long arm of chromosome 19 (1p/19q) (Sanson et al., 2009). Despite this new classification, heterogeneity within these subtypes and within individual tumors remains and cell populations with different mutations and expression profiles exist which are likely to have different malignancy characteristics (Patel et al., 2014). Identification of additional molecular characteristics of these subpopulations of cells would greatly benefit diagnostics.

For many years, tissue- and cell-specific expressions of intermediate filament proteins have been useful in tumor diagnostics (Dey, Togra, & Mitra, 2014). In 1971, upon the isolation of filaments from fibrous astrocytes, the 50 kDa type III intermediate filament glial fibrillary acidic protein (GFAP) was identified and characterized. One year later, high expression of GFAP in glioma with astrocyte characteristics, astrocytoma, was described for the first time, followed by many reports thereafter (Delpech et al., 1978; Uyeda, Eng, & Bignami, 1972; van der Meulen, Houthoff, & Ebels, 1978). Multiple studies characterized GFAP expression in glioma subtypes leading to the establishment...
of GFAP as a biomarker for astrocytoma that is still used to date (Dunbar & Yachnis, 2010). In the healthy human brain, GFAP is mainly expressed in mature astrocytes (Middeldorp & Hol, 2011). Therefore, in clinical as well as fundamental experimental settings, high GFAP expression is believed to mark more differentiated, less malignant tumors. However, more recently GFAP expression was observed in the radial glia of the developing human brain and in adult neural stem cells of the adult brain (Middeldorp & Hol, 2011; Roelofs et al., 2005; van den Berge et al., 2010), showing that GFAP is also expressed in immature, nondifferentiated CNS cells. Since then, GFAP is often used to mark cells with stem cell characteristics in glioma and to target neural stem cells to induce gliogenesis in animal models (Kwon et al., 2008; J. Chen et al., 2012; Bradshaw et al., 2016; Guichet et al., 2016; Kanabur et al., 2016; Jiang et al., 2017; Welker, Jaros, An, & Beattie, 2017). In addition, GFAP is up-regulated in non-neoplastic astrocytes that become reactive in response to the growth of the tumor and do not reflect the differentiation state of neoplastic cells (Gullotta, Schindler, Schmutzler, & Weeks-Seifert, 1985; Yoshii et al., 1992; H. Y. Yang, Lieska, Glick, Shao, & Pappas, 1993). Therefore, high GFAP levels in tumor specimens may not be a direct indication of a less malignant, more differentiated astrocytoma subtype. Indeed, our recent studies in which we determined the expression of different GFAP isoforms show that higher levels of the alternative splice variant GFAPδ relative to the canonical variant GFAPα are associated with a higher malignant and less differentiated astrocytoma subtype (Stassen et al., 2017). In vitro studies that show a higher malignant gene expression profile and changes in astrocytoma malignant behavior in cells with higher levels of GFAPδ relative to GFAPα, as observed in neurogenic stem cells of the healthy brain (Middeldorp & Hol, 2011; Roelofs et al., 2005; van den Berge et al., 2010), further support the hypothesis of GFAP as a marker of more than lower malignant astrocytoma (Moeton et al., 2014; Stassen et al., 2017).

In order to investigate this hypothesis, we systematically reviewed all existing literature on GFAP expression in patient material of astrocytoma (grade I–IV classified according to the WHO 2007 system or earlier), IDH1 wild-type (IDHwt) glioma, and IDH1 mutated glioma without a 1q19p codeletion (IDHmut noncodel; classified according to the WHO 2016 system). We included studies that determined the presence of GFAP in control brains and astrocytoma tissue, in astrocytoma of different malignancy grades, in different areas of the tumor, in blood of astrocytoma patients, in proliferating or invasive cells, and studies that describe the morphology of GFAP expressing cells. We conclude that a strong correlation of GFAP to astrocytoma malignancy is absent, that the GFAP positive population is highly heterogeneous, and that distinguishing between the GFAP isoforms GFAPδ and GFAPα might improve the assessment of the differentiation state and malignancy of the tumor and identify subpopulations of GFAP expressing astrocytoma cells.

2 | SEARCH STRATEGY

Collection of all literature on GFAP expression in astrocytoma patient material was systematically performed. The following code was used to search the PubMed database on August 4, 2015: “(((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((Astrocytoma[Mesh]) OR Astrocytoma[Title/Abstract]) OR astrocytomas[Title/Abstract]) OR glioblastoma[Title/Abstract]) OR glioblastomas[Title/Abstract]) OR astroglia[Title/Abstract]) OR astrogliomas[Title/Abstract]) OR astrocytic glioma[Title/Abstract]) OR astrocytic gliomas[Title/Abstract])]) OR astrocytic tumor[Title/Abstract]) OR astrocytic tumors[Title/Abstract])]) OR astrocytic tumor[Title/Abstract])]) OR gliosarcoma[Title/Abstract]) OR gliosarcomas[Title/Abstract]) OR glomatosis cerebri[Title/Abstract]) AND ((((((((((((("Glial Fibrillary Acidic Protein"-Mesh]) OR Glial Fibrillary Acidic Protein[Title/Abstract]) OR Glial Intermediate Filament Protein[Title/Abstract]) OR Gliarial Intermediate Filament Proteins[Title/Abstract]) OR Glial Fibrillary Acid Protein))))) OR astroprotein[Title/Abstract]) OR GFA protein[Title/Abstract]) OR GFAP[Title/Abstract]) NOT ("Animals"[Mesh] NOT ("Human"[Mesh] AND "Humans"[Mesh])").

This resulted in 1322 publications which were scanned on title and abstract by two researchers, independently. Case studies, animal, and in vitro studies were excluded. The 329 remaining studies were evaluated in detail and 77 studies that all analyzed GFAP expression levels or patterns in astrocytoma patient material of grade I, grade II, grade III, or grade IV (WHO classification of the respective year) were finally included. We searched PubMed again with the same search string on September 7, 2018, and an additional 11 papers were included.

3 | RESULTS

We categorized 88 studies that quantified or described GFAP mRNA or protein expression in human astrocytoma patient material based on the methods that were used and comparisons that were made (Tables 1–8). The results of the studies in each category are discussed below.

3.1 | GFAP expression is increased in astrocytoma versus healthy human CNS tissue

Two early studies already address one of the most important questions considering GFAP in astrocytoma; is the GFAP protein in astrocytoma different from GFAP in the healthy brain, in respect of its immunohistochemical characteristics and expression level? Delpech et al. (1978) used radial immunodiffusion analysis to demonstrate that GFAP in healthy human brain extracts is immunohistochemically similar to GFAP in astrocytoma extracts (Delpech et al., 1978). This agrees with the study of Dittmann et al. (1977) who show, by rocket immunoelectrophoresis, that there are no immunohistochemical differences between GFAP isolated from astrocytoma and the adult human brain. However, GFAP isolated from fetal human brain has different characteristics in this assay when compared to GFAP from astrocytoma or adult human brain (Dittmann et al., 1977). Both studies, and many studies that followed hereafter, show that GFAP expression in astrocytoma is increased compared to healthy brain tissue (Table 1) both at the protein (Delpech et al., 1978; Dittmann et al., 1977; Mauro et al., 1991; Narayan et al., 1986; Palfreyman et al., 1979) and mRNA (Bien-Moller et al., 2018; Laczkó et al., 2007) level. In contrast, a more recent study that used two-dimensional (2-DE) gel electrophoresis followed by mass spectrometry, shows a decrease in the 50 kDa canonical GFAP
protein in grade IV astrocytoma (although this protein is increased in grade III astrocytoma; Chumbalkar et al., 2005). Interestingly, a second GFAP protein of different mass and isoelectric point was detected in this study, which is upregulated in both grade III and grade IV astrocytoma. This suggests the expression of different GFAP proteins in these tumors, which potentially are isoforms, differently phosphorylated proteins, or degradation products. A variety of GFAP proteins with different molecular weights are described in a second 2-DE gel electrophoresis study that compares astrocytoma to healthy brain tissue, and the results show a higher expression of a 49 kDa GFAP protein and proteins ranging from 36 to 49 kDa in astrocytoma (Narayan et al., 1986). In a third study, 36 kDa and 50 kDa GFAP proteins are detected in astrocytoma, whereas only the 36 kDa GFAP protein is detected in control white matter tissue (Luider et al., 1999). Together, these studies show that the canonical GFAP protein is increased in astrocytoma compared to healthy brain tissue, but also that the detection of different GFAP forms (either splice isoforms, differentially phosphorylated forms, or degradation products) can be potentially used to discriminate between astrocytoma and healthy tissue, and astrocytoma of different malignancy.
For example, lower molecular weight GFAP fragments that result from caspase-mediated proteolysis are indicative of cellular stress and could be used as an additional biomarker for astrocytoma. These lower molecular weight GFAP fragments have been detected in brain extracts of Alexander disease patients, a rare nervous system disorder caused by GFAP mutations (M.-H. Chen, Hagemann, Quinlan, Messing, & Perng, 2013; Lin, Messing, & Perng, 2017). As specific antibodies that detect these fragments are available (M.-H. Chen et al., 2013; Lin et al., 2017), it might be interesting to use these to check for the presence of the caspase-cleaved fragments in astrocytoma as well.

### 3.2 | Inconsistent correlation of GFAP expression levels to astrocytoma malignancy

In Tables 2–4, we show all published results on general GFAP expression levels in astrocytoma grade I, II, III, and IV as determined by immunohistochemistry. Table 2 lists all studies that classified tumors of different grades as positive or negative for GFAP, without any form of quantification. Seventeen out of 20 studies show GFAP expression in all astrocytomas, although three of these 17 studies describe a focal or weaker expression in some tumors (Donev et al., 2010; Gullotta et al., 1985; Oh & Prayson, 1999). Only two out of the 20 studies specify the malignancy grade in which GFAP negative areas or tumors are found. Takenaka et al. (1985) describe GFAP negative tumors in astrocytoma of all grades (Takenaka et al., 1985). van der Meulen et al. (1978) describe GFAP negative tumors in astrocytoma of all grades (Takenaka et al., 1985). van der Meulen et al. (1978) describe GFAP immunoreactivity in all grade I and II astrocytomas, the presence of GFAP negative areas in grade III, and show that four out of 17 grade IV astrocytomas are GFAP negative. Quantification of differences in GFAP expression between astrocytoma grades was not performed in the studies listed in Table 2. Nevertheless, the observation of a decreasing number of GFAP positive cells with increasing astrocytoma grade is frequently mentioned (Cras et al., 1988; Cruz-Sanchez et al., 1992; Gullotta et al., 1985; Oh & Prayson, 1999; Roys et al., 1986; van der Meulen et al., 1978; Xing et al., 2016). One of the studies, however, describes stronger staining of GFAP in grade III compared to lower grade astrocytoma (Cruz-Sanchez et al., 1992).

| Histological grade | GFAP positive immunostaining                                                                 | Reference                                                                 |
|--------------------|-----------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Low and high grade (n = 15) | GFAP positive cells and processes in all astrocytoma.                                        | Chronwell, McKeever, & Kornblith, 1983                                   |
| Low and high grade (n = 13) | GFAP reactivity in all tumors.                                                                 | Royds, Ironside, Taylor, Graham, & Timperley, 1986                       |
| Low and high grade (n = 24) | GFAP reactivity in all tumors.                                                                 | Cras, Martin, & Gheuens, 1988                                            |
| Low and high grade (n = 66) | GFAP reactivity in all tumors.                                                                 | Cruz-Sanchez et al., 1992                                                |
| Grade I, II, III (n = 15) | GFAP reactivity in all tumors.                                                                 | Hashemi, Naderian, Kadivar, Nilipour, & Gheytanchi, 2014                 |
| Grade I, II, III (n = 10) | GFAP reactivity in all tumors.                                                                 | Kros, Van Eden, Stefanko, Waayer-Van, & van der Kwast, 1990             |
| Grade IV (n = 82) | Strong GFAP reactivity in 43/82 tumors and weaker in 39/82 tumors.                           | Donev, Scheithauer, Rodriguez, & Jenkins, 2010                          |
| Grade IV (n = 39) | GFAP reactivity was almost always observed.                                                   | Cuny et al., 2002                                                        |
| Grade IV (n = 26) | GFAP reactivity was almost always observed.                                                   | Sembritzi, Hagel, Lamszus, Deppert, & Bohn, 2002                        |
| Grade IV (n = 30) | GFAP reactivity in all tumors.                                                                | Terada, 2015                                                             |
| Low grade (n = 40), grade IV (n = 16) | GFAP reactivity in all tumors.                                                           | Goyal et al., 2015                                                       |
| Grade II (n = 43), grade III and IV (n = 33) | Only report decreasing GFAP levels with increasing grade.                      | Xing et al., 2016                                                        |

| n, number of cases.          |
Statistics were not performed or mentioned in these studies. et al., 2013) or the expression of different proteins including GFAP expression pattern of different intermediate filament proteins (Skalli et al., 2013). However, patients with the grade IV subtype that correlates with high GFAP expression as identified by Motomura et al. (2012), the astrocytic mesenchymal subtype, have significantly worse survival probability compared to the oligodendrocyte precursor subtype with low GFAP expression (Motomura et al., 2012). These results further emphasize the absence of a consistent correlation of GFAP expression to a more differentiated, lower malignant astrocytoma.

Table 5 lists the studies that quantified GFAP expression levels in astrocytoma homogenates. Four out of eight studies show no significant differences in GFAP protein or RNA levels between the different astrocytoma grades. Two studies that quantified RNA (Dmytrenko et al., 2009) or protein levels (Rasmussen et al., 1980) both report a high variability in expression. In two other studies that quantified protein levels, GFAP is higher in grade II compared to grade IV astrocytoma (Jacque et al., 1978; Odreman et al., 2005), which reached statistical significance when tested (Odreman et al., 2005). In addition, a third study reports higher GFAP levels in grade I and II compared to grade III and IV astrocytoma but only show western blot evidence for one sample of each grade (Peraud et al., 2003). In short, studies that analyzed astrocytoma homogenates neither generate consistent results on the correlation of GFAP expression to astrocytoma malignancy grades and additional analyses in larger cohorts are needed. In our own previously published study in which we used RNA sequencing data of 310 patients available at TCGA we do show a strong

| Histological grade | Scoring system | GFAP positive cell score | Reference |
|--------------------|---------------|--------------------------|-----------|
| Grade III (n = 1), Grade IV (n = 11) | 0, 5–25%, 25–50%, 75–100% | 0: - 5–25%: 2 grade IV 25–50%: 4 grade IV 75–100%: 5 grade IV, 1 grade III | Jones, Bigner, Schold Jr., Eng, & Bigner, 1981 |
| Grade IV (n = 97) | Not present, In single cells, In groups of cells, in more than 30% of cells | Not present: 1/97, Single cells: 16/97, Groups of cells: 20/97, More than 30% of cells: 60/97 | Schmidt et al., 2002 |
| Grade II (n = 5), Grade III (n = 10), Grade IV (n = 26) | Negative, single cells, cluster of cells (20–50%), 50%–90% of cells are positive, Almost 100% are positive | Grade I and II: >50% of the cells Grade III and IV: Single cells or negative | Peraud et al., 2003 |
| Grade I and II (n = 5), Grade IV (n = 4) | Scores from 0 to 3 | 9/9 score 2 or 3 | Tan, Magdalene Koh, & Tan, 2006 |
| Grade II (n = 10), Grade III (n = 11), Grade IV (n = 5) | Percentage of positive cells | All astrocytoma examined ranged from 5% to 100% of positive cells. | Rousseau et al., 2006 |
| Grade I (n = 8), Grade IV (n = 8) | No positive cells, 1–10% of positive cells, 11–25%, 26–50%, 51–90%, 91–100% | Grade I: 8/8 26–50% Grade IV: 8/8 51–100% | Colin et al., 2007 |
| Grade I (n = 15), Grade II (n = 26), Grade III (n = 4), Grade IV (n = 8) | 0, 10%, 11–50%, 50–75%, >75% of positive cells Weak, medium, strong intensity | Grade I: 15/15 > 75% Grade II: 24/26 > 75%, 2/26 50–75% Grade III: 4/4 > 75% Grade IV: 7/8 75%, 1/8 50–75% GFAP intensity was strong in all cases. | Shuangshoti et al., 2009 |
| Grade II (n = 25), Grade III (n = 29), Grade IV (n = 41) | <5%, >5% | Grade II: 25/25 > 5% Grade III: 26/29 > 5% Grade IV: 39/41 > 5% | Liu, Lu, Ohgaki, Merlo, & Shen, 2009 |

Studies in which a scoring system is used to quantify the level of GFAP immunostaining are listed in Tables 3 and 4. In Table 3, data of studies are shown in which GFAP is quantified without applying statistics to test for the significance of differences observed between astrocytomas. In six out of eight of these studies, there are no clear differences between GFAP scores in low- and high-grade astrocytoma. One study reports lower GFAP immunostaining scores in grade III and IV compared to I and II (Peraud et al., 2003) and in contrast, another study describes higher GFAP scores in grade IV compared to grade I astrocytoma (Colin et al., 2007). In the studies listed in Table 4, statistical testing is used to determine the significance of the differences in GFAP immunostainings between astrocytoma of different grades. Two out of 11 studies that compare grade I and II to grade III and grade IV, show significant higher GFAP scores in grade I and II astrocytoma (Hlobilkova et al., 2007; L. Yang et al., 2014). In one study, a significant decrease in GFAP levels with increasing astrocytoma grade is reported (Laczko et al., 2007) and a fourth study finds a significant correlation of GFAP levels to malignancy grade and a significant difference between grade II and IV astrocytoma (Schwab et al., 2018). Seven out of the 11 studies failed to show significant differences in the percentage of GFAP positive cells between astrocytoma of different grades of malignancy. Based on these studies that used immunohistochemistry analyses, we conclude that GFAP protein levels or the number of GFAP positive cells do not correlate to astrocytoma malignancy grade.

Within grade IV astrocytoma, GFAP has been associated with specific grade IV subtypes which are determined based on either the expression pattern of different intermediate filament proteins (Skalli et al., 2013) or the expression of different proteins including GFAP (Motomura et al., 2012). There is no significant difference in the survival probability of patients with either of the grade IV subtypes that were identified based on intermediate filament protein expression (GFAP, vimentin, nestin, and synemin; Skalli et al., 2013). However, patients with the grade IV subtype that correlates with high GFAP expression as identified by Motomura et al. (2012), the astrocytic mesenchymal subtype, have significantly worse survival probability compared to the oligodendrocyte precursor subtype with low GFAP expression (Motomura et al., 2012). These results further emphasize the absence of a consistent correlation of GFAP expression to a more differentiated, lower malignant astrocytoma.

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The above-described studies show a large variation in outcomes. To a certain extent, variation between studies is explained by the different methods of analysis, grouping of patient samples, sample sizes, scoring systems, antibodies used, quality of tissues, and variation in staining intensity within and between tumors (Cuny et al., 2002). Moreover, the complexity of GFAP positive cell morphology and staining patterns hamper quantification of immunohistochemistry data of the number of GFAP positive cells to the total number of cells (Tanaka et al., 2008).

Besides these methodological issues, the inconsistent correlation of GFAP expression to astrocytoma malignancy might result from decrease in general GFAP mRNA levels in grade IV compared to grade II and grade III astrocytoma (Stassen et al., 2017).
heterogeneity in localization and function of GFAP positive astrocytoma cells within and between tumors. Local variability of GFAP immunostaining was highlighted in many studies (Cras et al., 1988; Cruz-Sanchez et al., 1992; Gullotta et al., 1985; Herpers et al., 1986; Royds et al., 1986; Sharpe & Baskin, 2016; Tascos et al., 1982; van der Meulen et al., 1978; Velasco et al., 1980). Thus, as was already noted in 1985 (Gullotta et al., 1985) and many times thereafter (Hashemi et al., 2014; Sembritzki et al., 2002) analysis of different areas of the same tumor is necessary to determine GFAP expression levels of a tumor. This is emphasized by two studies that specifically reporting on shorter processes in grade III and grade IV astrocytoma (Zamecnik et al., 2004). However, as GFAP positive neoplastic cells are intermingled with GFAP positive reactive astrocytes, different areas of the same tumor are described to be negative for GFAP, although weak GFAP specific staining of processes (Peraud et al., 2003; Zamecnik et al., 2004) and a dense fibrillary network with processes, bipolar elongated cells, cells arranged in parallel bundles, stellate/polygonal cells, multinucleated cells, cells associated with the vasculature, and cells expressing GFAP aligning the inner surface of the plasma membrane only. GFAP positive immunostaining ranges from a dense meshwork of GFAP positive processes without a clear visible cell body to areas with single large gemistocytic cells with strong cytoplasmic staining and short processes. Microcystic areas within the tumor are described to be negative for GFAP, although weak GFAP staining in these areas has been described as well. While these characteristics are observed in all astrocytoma grades, some have been observed more frequently in low- or high-grade astrocytoma. GFAP negative cytoplasmic (Gullotta et al., 1985; Velasco et al., 1980) and necrotic areas (Tascos et al., 1982), and focal GFAP staining are more often seen in higher grade astrocytoma (Cras et al., 1988; Cruz-Sanchez et al., 1992), whereas diffuse GFAP staining (Cruz-Sanchez et al., 1992; Royds et al., 1986) and a dense fibrillary network with clear staining of processes (Peraud et al., 2003; Zamecnik et al., 2004) are more often seen in lower grade astrocytoma with one study specifically reporting on shorter processes in grade III and grade IV astrocytoma (Zamecnik et al., 2004). However, as GFAP positive neoplastic cells are intermingled with GFAP positive reactive astrocytes, determining the neoplastic origin of these thin and complex processes is

Many studies have described large heterogeneity in immunostaining characteristics and the morphology of GFAP positive cells (Table 6). In general, GFAP immunofluorescence is found in the cell body and processes of neoplastic cells and these immunostainings clearly visualize cell morphology. More specifically, GFAP is expressed in gemistocytic cells with uniform staining in cell bodies with shorter processes, bipolar elongated cells, cells arranged in parallel bundles, stellate/polygonal cells, multinucleated cells, cells associated with the vasculature, and cells expressing GFAP aligning the inner surface of the plasma membrane only. GFAP positive immunostaining ranges from a dense meshwork of GFAP positive processes without a clear visible cell body to areas with single large gemistocytic cells with strong cytoplasmic staining and short processes. Microcystic areas within the tumor are described to be negative for GFAP, although weak GFAP staining in these areas has been described as well. While these characteristics are observed in all astrocytoma grades, some have been observed more frequently in low- or high-grade astrocytoma. GFAP negative cytoplasmic (Gullotta et al., 1985; Velasco et al., 1980) and necrotic areas (Tascos et al., 1982), and focal GFAP staining are more often seen in higher grade astrocytoma (Cras et al., 1988; Cruz-Sanchez et al., 1992), whereas diffuse GFAP staining (Cruz-Sanchez et al., 1992; Royds et al., 1986) and a dense fibrillary network with clear staining of processes (Peraud et al., 2003; Zamecnik et al., 2004) are more often seen in lower grade astrocytoma with one study specifically reporting on shorter processes in grade III and grade IV astrocytoma (Zamecnik et al., 2004). However, as GFAP positive neoplastic cells are intermingled with GFAP positive reactive astrocytes, determining the neoplastic origin of these thin and complex processes is

| Histological grade | Method | GFAP expression levels | Significant effect? | Reference |
|--------------------|--------|------------------------|---------------------|-----------|
| Protein            |        |                        |                     |           |
| Grade II (n = 3), Grade III (n = 1), Grade IV (n = 7) | Rocket immuno-electrophoresis | Grade II: >12 μg/mg | NA        | Jacque et al., 1978 |
| Grade III (n = 5), Grade IV (n = 8) | Radial immunodiffusion | No difference between grades. | No       | Delpech et al., 1978 |
| Low grade (n = 4), Grade IV (n = 6) | Quantitative immune-electrophoresis | Variation in levels, no correlation to malignancy grade. | No       | Rasmussen, Bock, Warecka, & Althage, 1980 |
| One sample of each grade | Western blot (to confirm staining data) | Decreased GFAP levels in grade III and IV compared to grade I and II. | NA       | Peraud et al., 2003 |
| Grade II (n = 10), Grade IV (n = 10) | 2-DE gel electrophoreses and mass spectrometry | Higher levels of GFAP in grade II compared to grade IV extracts. GFAP was one of the 15 proteins that was differentially expressed. | Yes      | Odreman et al., 2005 |
| RNA                |        |                        |                     |           |
| Grade I and II (n = 6), Grade III (n = 9), Grade IV (n = 20) | Northern blot analysis, 2,600 bp probe | No difference between astrocytoma grades. | No       | Mauro et al., 1991 |
| Low grade (n = 7), Grade III (n = 9), Grade IV (n = 14) | Northern blot analysis | No difference between astrocytoma grades. | NA       | Dmytrenko et al., 2009 |
| Grade II (n = 55), Grade III (n = 105), Grade IV (n = 150) | RNA sequencing data; normalized gene expression values (the cancer genome atlas) | Higher levels in grade II and III astrocytoma compared to grade IV. | Grade II versus IV: FDR = 1.58E–10 | Stassen et al., 2017 |

FDR, false discovery rate; NA, not available (authors did not perform statistics); n, number of cases.

*p values are given if provided.

### Table 6 GFAP quantification in astrocytoma homogenates

- **Protein**
  - **Grade II (n = 3), Grade III (n = 1), Grade IV (n = 7)**: Rocket immuno-electrophoresis
    - **Grade II**: >12 μg/mg
    - **Grade III**: 47.5 μg/mg (n = 1)
    - **Grade IV**: <2 μg/mg
  - **Grade III (n = 5), Grade IV (n = 8)**: Radial immunodiffusion
    - No difference between grades.
  - **Low grade (n = 4), Grade IV (n = 6)**: Quantitative immune-electrophoresis
    - Variation in levels, no correlation to malignancy grade.
  - **One sample of each grade**: Western blot (to confirm staining data)
    - Decreased GFAP levels in grade III and IV compared to grade I and II.
  - **Grade II (n = 10), Grade IV (n = 10)**: 2-DE gel electrophoreses and mass spectrometry
    - Higher levels of GFAP in grade II compared to grade IV extracts. GFAP was one of the 15 proteins that was differentially expressed.

- **RNA**
  - **Grade I and II (n = 6), Grade III (n = 6), Grade IV (n = 20)**: Northern blot analysis, 2,600 bp probe
    - No difference between astrocytoma grades.
  - **Low grade (n = 7), Grade III (n = 9), Grade IV (n = 14)**: Northern blot analysis
    - No difference between astrocytoma grades.
  - **Grade II (n = 55), Grade III (n = 105), Grade IV (n = 150)**: RNA sequencing data; normalized gene expression values (the cancer genome atlas)
    - Higher levels in grade II and III astrocytoma compared to grade IV.
| Histological type | Described patterns (1-15) | Described differences between astrocytoma grades | Reference |
|-------------------|---------------------------|-----------------------------------------------|-----------|
| Grade III (n = 5), Grade IV (n = 8) | 1. Immunofluorescence found in the cell body and processes of malignant cells. 7. Peri-vascular staining. 8. GFAP expression aligning the inner surface of the plasma membrane. | – | Delpech et al., 1978 |
| Astrocytoma (n = 4), Grade IV (n = 8) | 1. Immunofluorescence found in the cell body and processes of malignant cells. 8. GFAP expression aligning the inner surface of the plasma membrane. 9. High GFAP expressing gemistocytic cells. 10. Uniform GFAP expression with extensions into short processes. 11. High GFAP expression in stellate-shaped elongated, bipolar, piloid cells. | – | Duffy, Huang, Rapport, & Graf, 1980 |
| Low grade (n = 17), Grade III (n = 16), Grade IV (n = 7) | 1. Immunofluorescence found in the cell body and processes of malignant cells. 2. Dense meshwork of GFAP positive cellular processes, stained over their full length, including thin processes. 3. GFAP negative microcystic (small cell) areas. 10. Uniform GFAP expression with extension into short processes. 9. High GFAP expressing gemistocytic cells. 11. Uniform GFAP expression with extensions into short processes. 12. GFAP expressing cells arranged in parallel bundles. 13. GFAP expression in multinucleated cells. 14. GFAP expression in the rim of Rosenthal fibers. | 3. More often observed in higher grade astrocytoma | Velasco, Dahl, Roessmann, & Gambetti, 1980 |
| Grade I (n = 5), Grade II and III (n = 27), Grade IV (n = 10) | 1. Immunofluorescence found in the cell body and processes of malignant cells. 3. GFAP negative microcystic (small cell) areas. 9. High GFAP expressing gemistocytic cells. 13. GFAP expression in multinucleated cells. 15. Necrotic GFAP negative areas. | 13. Observed in grade IV astrocytoma 15. Observed in grade IV astrocytoma | Tascos, Parr, & Gonatas, 1982 |
| Low and high grade (n = 15) | 7. Peri-vascular staining. | – | Chronwall et al., 1983 |
| Grade I, II, and III (n = 58) | 3. GFAP negative microcystic (small cell) areas. | 3. More often observed in higher grade astrocytoma | Gullotta et al., 1985 |
| Astrocytoma (n = 15) | 1. Immunofluorescence found in the cell body and processes of malignant cells. 3. GFAP negative microcystic (small cell) areas. 9. High GFAP expressing gemistocytic cells. 11. High GFAP expression in stellate-shaped elongated, bipolar, piloid cells. 12. GFAP expressing cells arranged in parallel bundles. 13. GFAP expression in multinucleated cells. 14. GFAP expression in the rim of Rosenthal fibers. | – | Smith & Lantos, 1985 |
| Low and high grade (n = 13) | 6. Diffuse/homogeneous GFAP staining | 6. More often in low-grade astrocytoma | Royds et al., 1986 |
| Astrocytoma (n = 71) | 2. Dense meshwork of GFAP positive cellular processes, stained over their full length, including thin processes. 7. Peri-vascular staining. | – | Herpers et al., 1986 |
| Grade I (n = 12), Grade III (n = 9), Grade IV (n = 24), | 1. Immunofluorescence found in the cell body and processes of malignant cells. 4. Focal GFAP expression only. 7. Peri-vascular staining. | 4. More often seen in grade III and specifically grade IV astrocytoma | Cras et al., 1988 |
| Low and high grade (n = 66) | 4. Focal GFAP expression only. 6. Diffuse/homogeneous GFAP staining. | 4. More often in high astrocytoma. 6. More often in low-grade astrocytoma. | Cruz-Sanchez et al., 1992 |
| Grade IV (n = 23) | 4. Focal GFAP expression only. | – | Oh & Prayson, 1999 |
| Grade I (n = 8), Grade II (n = 18), Grade III (n = 4), Grade IV (n = 30) | 6. Diffuse/homogeneous GFAP staining. | 6. More often seen in grade IV compared to grade I and II | Katsetos et al., 2001 |
| Grade IV (n = 39) | 1. Immunofluorescence found in the cell body and processes of malignant cells. 5. GFAP positive large-sized cells with numerous processes but variable staining intensity. | – | Curry et al., 2002 |
| Grade II (n = 5), Grade III (n = 10), Grade IV, (n = 26) | 1. Immunofluorescence found in the cell body and processes of malignant cells. 9. High GFAP expressing gemistocytic cells. | 1. Staining of processes more often observed in low-grade astrocytoma | Peraud et al., 2003 |

(Continues)
The level of GFAP expression in proliferating and invading astrocytoma cells, GFAP is expressed in cells with different biological functions that contribute to tumor malignancy. The degree of proliferation and invasion are the two most important traits of astrocytoma malignancy, and we examined the publications that describe the level of GFAP expression in proliferating and invading cells in human astrocytoma material. Two studies report absence of GFAP expression in proliferating cells as shown by the lack of GFAP expression in mitotic cells in astrocytoma of all grades (Schiffer, Giordana, Germano, & Mauro, 1986) and a lack of GFAP coexpression with the proliferation marker Ki67 in 15 low grade and 10 grade IV astrocytomas (Kros, Schouten, Janssen, & van der Kwast, 1996). In contrast, low levels of proliferating GFAP-expressing cells are detected in another study that reports coexpression of GFAP and Ki67 in 8.8% (±13.6%) of the total number of Ki67 expressing cells in low-grade astrocytoma compared with high-grade astrocytoma (L. Yang et al., 2014). According to these studies, GFAP is expressed in both proliferating and nonproliferating cells. Local differences in the distribution of these cells might again account for the variation in observations. As mentioned previously, GFAP is expressed at different levels in the core, intermediate transitional area, and periphery of the tumor (Pistollato et al., 2010). Analysis of stem cell and proliferation markers in these areas shows that the core mainly consists of CD133 positive and O6-methylguanine-DNA methyltransferase promoter methylation positive (MGMT+) stem cells that can form small neurospheres in culture. The highest level of Ki67 expression is observed in the intermediate area and these cells show the highest growth potential in vitro. Cells in the periphery show the lowest Ki67 expression and lowest growth potential in vitro. These cells do not grow as neurospheres but show a differentiated morphology. In vitro, GFAP expression does not significantly differ between cells cultured from the different areas of a tumor, although a trend for increased GFAP expression in peripheral cells is observed (Pistollato et al., 2010).

Another study reports on coexpression of the stem cell marker CD133 with GFAP (Tamura et al., 2013) further emphasizing that GFAP expression does not solely mark proliferating or nonproliferating, differentiated or stem-cell like cells in astrocytoma patient material.

Three studies determined GFAP expression in invading parts of astrocytoma. In areas of grade IV astrocytoma that invade cortical and white matter tissue, both GFAP positive and GFAP negative cells are present (Schiffer et al., 1986). In addition, invading astrocytoma cells into connective tissue (i.e., meningeal invasion) are marked by increased GFAP expression in the invading part compared to the non-invading part (Herpers, Budka, & McCormick, 1984; Nakopoulou et al., 1990). These studies further emphasize the large variation in GFAP expressing cells in astrocytoma that most likely causes the lack of a strong correlation of GFAP to astrocytoma malignancy based on current published literature. The identification of GFAP expressing subtypes of cells will be necessary to determine the role of GFAP in astrocytoma malignancy.

### 3.4 Differential GFAP isoform expression to distinguish astrocytoma subtypes

In the healthy human brain, the GFAP positive subtype of neurogenic stem-cell like cells can be distinguished by the expression of a GFAP isoform that results from the process of alternative splicing. GFAP6
find differences in GFAP expression between astrocytoma grades to control tissue (Blechingberg et al., 2007). Interestingly, studies that decreased levels in five out of eight grade IV astrocytoma compared GFAP et al., 2009; Heo et al., 2012). In contrast, RNA quantification of toma of all grades when analyzed by immunohistochemistry (Choi reactive gliosis (Andreiuolo et al., 2009), and is increased in astrocytoma healthy brain (Choi et al., 2009; Heo et al., 2012), but is detected in GFAP 2010). A few studies have determined the expression level of the (Middeldorp & Hol, 2011; Roelofs et al., 2005; van den Berge et al., 2010). A few studies have determined the expression level of the GFAPδ isoform in astrocytoma of different grades as well. The studies in Table 7 report that GFAPδ expression is rarely observed in the healthy brain (Choi et al., 2009; Heo et al., 2012), but is detected in reactive gliosis (Andreiuolo et al., 2009), and is increased in astrocytoma of all grades when analyzed by immunohistochemistry (Choi et al., 2009; Heo et al., 2012). In contrast, RNA quantification of GFAPδ shows increased levels in only three out of eight and decreased levels in five out of eight grade IV astrocytoma compared to control tissue (Blechingberg et al., 2007). Interestingly, studies that find differences in GFAP expression between astrocytoma grades report on a decrease of general GFAP levels with increasing astrocytoma grade, but higher levels of GFAPδ are found in grade IV astrocytoma compared to grade I (Andreiuolo et al., 2009), grade II (Brehar et al., 2014), and grade I, II, and III (Choi et al., 2009). In one of these studies, general GFAP levels are quantified and show a decrease in grade IV compared to grade III, II, I, and control tissue (Choi et al., 2009). In grade I, II, and III spinal cord astrocytoma, GFAPδ expression also increases with increasing grade (Heo et al., 2012). Interestingly, GFAPδ levels are significantly associated with a rounder cell morphology and fewer cellular processes (Choi et al., 2009; Heo et al., 2012), and with highly invasive grade IV astrocytoma (Brehar et al., 2014). In addition, two grade II astrocytoma that are categorized as highly

| Histological type | Methods | GFAP isoform expression | Reference |
|-------------------|---------|-------------------------|----------|
| Grade III (n = 8), Grade IV (n = 1), Frontal cortex control (n = 1) | Quantitative PCR | GFAPα, GFAPδ, and GFAPδ expression is decreased in 5/8 and increased in 3/8 astrocytoma compared to control tissue. The GFAPδ/GFAPα ratio is increased in all astrocytoma compared to control tissue. | Blechingberg et al., 2007 |
| Grade I (n = 4), Grade IV (n = 2), Control healthy and epileptic tissue | Immunohistochemistry; No quantification | GFAPδ is coexpressed with GFAP in reactive astrocytes. GFAPδ expressed in 1/4 grade I astrocytoma, focal expression in 3/4 grade I tumors, but mostly negative. Grade IV: Strong focal GFAPδ expression | Andreiuolo et al., 2009 |
| Grade I (n = 3), Grade II (n = 5), Grade III (n = 4), Grade IV (n = 4), Control autopsy material (n = 4) | Immunohistochemistry; Quantification of mean grey value after outlining the cell | Increased pan GFAP and GFAPδ expression in astrocytoma compared to control tissue. Pan GFAP levels significantly increase from grade I to grade III and are decreased in grade IV astrocytoma. GFAPδ expression is mostly undetectable in control cells and increases with increasing astrocytoma grade. GFAP positive control cells are stellate-shaped with well-developed processes. Grade I and II: Stellate polygonal or round cells Grade III and IV: Round and spindle shaped cells GFAPδ is mainly observed in cell bodies not in processes. Inverse correlation of GFAPδ expression intensity and the amount of processes (round>polygonal>stellate). | Choi, Kwak, Kim, Sheen, & Kang, 2009 (same lab as Heo et al., 2012) |
| Spinal cord astrocytoma Grade I (n = 3), Grade II (n = 14), Grade III (n = 5) | Immunohistochemistry; Quantification of mean grey value after outlining the cell | Increased GFAPδ expression in spinal cord astrocytoma compared to control tissue. Strong positive correlation of GFAPδ with astrocytoma grade. Weaker positive correlation of pan GFAP with astrocytoma grade. Grade I and II: GFAPδ expression in stellate polygonal or round cells. Grade III: GFAPδ expression in round and spindle-shaped cells. | Heo et al., 2012 (same lab as Choi et al., 2009) |
| Grade II (n = 7), Grade III (n = 2), Grade IV (n = 35) | Immunohistochemistry; Quantification of GFAPδ positive cell number Analysis of MRI scans: High invasive: Corpus callosum infiltration with opposite hemisphere invasion and multicentric astrocytoma with tumor foci in both hemispheres Low invasive: Unifocal deep-seated astrocytoma | 7/7, 2/2 and 34/35 astrocytoma express GFAPδ Significant increase in GFAPδ expression in grade IV (83.8%) compared to grade II (57.1%). Significant correlation of GFAPδ to neuroimaging invasiveness. Two low-grade invasive tumors show high levels of GFAPδ. | Brehar, Arsene, Brinduse, & Gorgan, 2014 |
| Grade II (n = 55), Grade III (n = 105), Grade IV (n = 150) | RNA sequencing data; normalized isoform expression values (the cancer genome atlas) | Significant decrease in the level of GFAPα in grade IV astrocytoma and a significant increase in the relative level of GFAPδ to GFAPα in grade IV astrocytoma. | Stassen et al., 2017 |

n = number of cases.
## Table 8: GFAP levels in body fluids of astrocytoma patients

| Histological grade | Method | Results | Significant effect? \(^a\) | Reference |
|--------------------|--------|---------|-----------------------------|-----------|
| Low grade (n = 3), Grade IV (n = 2) | GFAP levels in cerebrospinal fluid (CSF) of patients by radiolabeling of GFAP with Iodine-125 | High levels of GFAP in CSF can discriminate between astrocytoma and other types of tumors and controls. | NA | Szymas, 1985 |
| Grade II (n = 13), Grade III (n = 14), Grade IV (n = 50), Healthy controls (n = 50) | GFAP levels in serum determined by ELISA | Detected in 40/50 grade IV astrocytoma. Higher levels compared to other tumors and controls. Correlation of GBM patient serum GFAP with: - Tumor volume, - Tumor necrosis volume, and - Number of necrotic GFAP positive cells. | Yes, p < 0.0001 | Jung et al., 2007 |
| Grade IV (n = 61), Healthy controls | GFAP positive circulating microparticles (MP) in blood detected by flow cytometry before and after surgery | Baseline: Baseline MP level higher in grade IV versus control. Increase in GFAP positive MPs after 7 days that persist up to 7 months after all surgeries (1 month and 4 months measurements as well). Higher number of GFAP positive MPs at 7 months compared to 7 days for subtotal resections only. Increased GFAP positive MPs after 7 months compared to 4 months in patients with radiological disease progression. | Yes, p = 0.05 | Sartori et al., 2013 |
| Grade II (n = 7), Grade III (n = 10), Grade IV (n = 34), Intercranial metastasis (ICM) (n = 41), MS patients (n = 25), Healthy controls (n = 26) | GFAP levels in serum determined by ELISA | GFAP detected in 0/7 grade II, 0/10 grade III, 13/34 grade IV, 1/41 metastasis, 1/25 MS patients, and 1/26 healthy controls. Significant association with grade IV astrocytoma diagnosis. Higher serum levels in grade IV compared to ICM. Grade IV: No correlation to neuroradiological characteristics. Patients with detectable GFAP in serum levels had a longer survival probability (11.3 months) compared to others (5.2 months). | Yes, p < 0.001 | Ilhan-Mutlu et al., 2013 |
| Grade IV (n = 141), Noncancer controls (n = 23), Brain metastasis (n = 5) | Isolation of mononucleated cells from peripheral blood and immunocytochemistry of cells | GFAP positive cells were detected in: 29/141 grade IV, 0/23 healthy controls, 1/5 brain metastasis. GFAP positive cells were more often detected in patients with EGFRvIII mutated tumors. There was no correlation to survival. | Yes | Muller et al., 2014 |
| Grade IV (n = 111), healthy controls (n = 99), nonglial brain tumors (n = 40) | GFAP levels in serum determined by ELISA | Higher levels in grade IV compared to healthy controls. Higher levels in grade IV compared to nonglial tumors. Detected in 30/111 patients. Larger tumor volume in patients with detectable compared to undetectable GFAP levels. No correlation to PFS or survival. | Yes, p < 0.01; Yes, p = 0.04; Yes, p < 0.001; NA | Gállego Pérez-Larraya et al., 2014 |
| Grade II (n = 11), Grade IV (n = 23), Healthy controls (n = 15) | GFAP levels in serum determined by ELISA | Above detection level in 2/15 healthy controls, 3/11 grade II, and 9/23 grade IV astrocytoma patients. Higher levels in grade IV patients. | No | Lange et al., 2014 |
| Grade I (n = 3), Grade II (n = 5), Grade III (n = 3), Grade IV (n = 25), Brain metastasis (n = 24), Healthy individuals (n = 132) | GFAP levels in serum determined by ELISA and immunohistochemistry of tumor tissue. | Higher GFAP levels in grade IV glioma compared to all others. Higher level of GFAP expression in the tumor subgroup with high GFAP serum levels. | p < 0.001; p = 0.035 | Tichy et al., 2016 |
| Recurrent tumors: Grade IV IDHmut (n = 4), Grade IV IDHwt (n = 3), Grade III (n = 3), Healthy controls (n = 3) | GFAP positive CD9+ exosomes in serum of patients determined by flow cytometry | GFAP positive CD9+ exosomes of total CD9+ exosomes were 7.9 times higher in patients with recurrent glioma (22.8%; 18.2–27.1%) compared to healthy controls (2.9%; 2.7–3.2%) at baseline. | NA | Galbo et al., 2017 |
invasive show strong GFAP\(\delta\) expression (Brehar et al., 2014). These studies indeed indicate that GFAP\(\delta\) can be used to identify astrocytoma subpopulations of cells as well and suggest that GFAP expressing cells with different functions (e.g., proliferating, quiescent, invasive, and static) consist of a different combination of GFAP protein isoforms. The detection of a second GFAP alternative splice variant, GFAP\(\kappa\), in RNA isolated from grade IV astrocytoma further supports this hypothesis (Bleichberg et al., 2007). We recently showed that quantification of the relative level of GFAP\(\kappa\) to GFAP\(\delta\), the GFAP\(\delta\)/GFAP\(\kappa\) ratio, using RNA sequencing data obtained from the cancer genome atlas (TCGA) indeed indicates that low- and high-grade astrocytoma express different combinations of GFAP variants. In astrocytoma grade IV, the GFAP\(\delta\)/GFAP\(\kappa\) ratio was significantly higher compared to grade II and III (WHO 2007; Stassen et al., 2017).

3.5 | GFAP as a blood biomarker

The most consistent results on the relationship of GFAP to astrocytoma malignancy have been generated by the analysis of blood or cerebrospinal fluid (CSF) of astrocytoma patients. An early study already shows that GFAP levels in CSF can be used to distinguish astrocytoma from other types of tumors and healthy controls (Szymas, 1985), but there was no follow up study. As shown in Table 8, subsequent studies link GFAP detection in blood to grade IV astrocytoma specifically. Eight out of 12 studies report on a significant association of GFAP levels detected in serum (Baumgarten et al., 2018; Gallego Pérez-Larraya et al., 2014; Ilhan-Mutlu et al., 2013; Jung et al., 2007; Kiviniemi et al., 2015; Tichy et al., 2016), in microparticles (Sartori Pérez-Larraya et al., 2014; Ilhan-Mutlu et al., 2013; Jung et al., 2007), from blood of grade IV astrocytoma patients in comparison to lower grade astrocytoma, nonglial tumors, other neurological diseases, and healthy controls. One of these studies reports detectable GFAP levels in blood of grade III astrocytoma patients as well, but levels in grade IV astrocytoma patients were significantly higher (Kiviniemi et al., 2015). Similarly, GFAP positive exosome numbers are increased in grade III and IV astrocytoma compared to healthy controls (Galbo et al., 2017). Two of the 12 studies report on higher GFAP levels in plasma of grade IV patients, but no statistics were performed (Vietheer et al., 2017) or no statistical significance was reached (Lange et al., 2014). High GFAP levels in blood of grade IV astrocytoma patients is in contrast with the most often described lower GFAP levels in higher grade astrocytoma tissue (Tables 3–5). However, although one study also finds higher GFAP expression levels in tumor cells of patients with high GFAP serum levels (Tichy et al., 2016),

### TABLE 8 (Continued)

| Histological grade | Method | Results | Significant effect? | Reference |
|-------------------|--------|---------|---------------------|-----------|
| Low grade (n = 2), Grade IV IDHwt (n = 29), Grade IV IDHmut (n = 1), Unknown (n = 3) | GFAP levels in serum determined by a sandwich immunoassay | 14 of 33 grade IV glioma patients showed high GFAP levels prior to surgery. 6 weeks after surgery GFAP levels in GFAP positive patients were decreased compared to levels prior to surgery. No increase in any of the patients. No correlation with tumor volume, survival or PFS. | NA | Vietheer et al., 2017 |
| Grade IV (n = 25), Brain metastasis (n = 7) | GFAP levels in serum determined by ELISA | Increase in GFAP levels directly after surgery. No further increase up to 7 days after surgery. Trend for increase after surgery for brain metastasis also observed. Higher levels in grade IV glioma compared to brain metastasis prior to surgery. No correlation to tumor volume. | \(p = 0.0172\) | Baumgarten et al., 2018 |
| Grade III (n = 13), Grade IV (n = 14), Healthy controls (n = 13) | GFAP levels in serum determined by ELISA | Preoperative: Higher levels of GFAP in grade IV compared to grade III. Higher levels of GFAP in grade IV compared to controls. Correlation to enhancing tumor volume (primary/recurrence). Correlation to necrotic tumor volume (primary/recurrence). Lower levels in IDHmut glioma. Correlation to Ki67 expression. No correlation of GFAP expression in tumor to serum levels. High GFAP levels related to poor PFS in primary tumors. Postoperative: Increased GFAP levels in 65% of glioma patients. No correlation to tumor volume. No difference between day 2, 3, 4, and 5 postsurgery. | \(p = 0.003\) | Kiviniemi et al., 2015 |

NA, not available (authors did not perform statistics); PFS, progression free survival; IDHwt, IDH1 wild-type; IDHmut, IDH1 mutated; n, number of cases; EGFRvIII, epidermal growth factor receptor gene amplification.

\(p\) values are given if provided.
another study reports on the absence of a correlation (Kiviniemi et al., 2015) suggesting that GFAP serum levels not directly reflect GFAP expression in the tumor. Indeed, multiple other tumor-related factors are associated with increased GFAP serum levels. A significant correlation to tumor volume and/or necrosis (Gállego Pérez-Larraya et al., 2014; Jung et al., 2007; Kiviniemi et al., 2015) and the number of GFAP positive necrotic cells (Jung et al., 2007) has been described, although in other studies a correlation to tumor volume was assessed but absent (Baumgarten et al., 2018; Ilhan-Mutlu et al., 2013; Vietheer et al., 2017). In addition, increased GFAP serum levels are observed up to 7 days after surgical removal of grade IV and III astrocytoma (Baumgarten et al., 2018; Kiviniemi et al., 2015). These studies suggest that GFAP serum levels are related to brain damage and cell death induced by, in, or near the tumor. This is supported by studies that have linked high GFAP serum levels in patients with traumatic brain injury (Bazarian et al., 2018; Thelin et al., 2017). Increased levels of GFAP positive microparticles can be observed from 7 days up to 7 months after surgery (Sartori et al., 2013), although another study that measured GFAP serum levels 6 weeks after surgery, does not show an increase in GFAP and for some patients the GFAP serum levels were even lower compared to levels before surgery (Vietheer et al., 2017). GFAP levels in blood might be induced by regrowth of the tumor, as levels of GFAP microparticles at 7 months compared to
4 | CONCLUSION

GFAP positive cells are present in tumors of all malignancy grades with a tendency for decreased GFAP levels with increasing astrocytoma grade. However, in current literature, a significant correlation is not consistently reproduced mainly caused by intra- and inter-tumor heterogeneity of GFAP positive cell localization, morphology, function, and expression of GFAP variants. Different types of evidence support the presence of a specialized GFAP intermediate filament network composed of different GFAP variants (splice isoforms, posttranslational modifications, degradation products) in astrocytoma cell subpopulations. As these variants, as shown for GFAPδ, differentially correlate to the malignancy of the tumor, the current use of commercial GFAP antibodies that recognize all isoforms most likely masks a consistent correlation of GFAP to astrocytoma malignancy grade. Discrimination between GFAP variants, as we show here for GFAPδ and GFAPα, helps to identify different types of GFAP positive cells that could improve the assessment of astrocytoma differentiation and malignancy. We hypothesize, as summarized in Figure 1, that GFAP is expressed in heterogeneous astrocytoma cells with a low malignant, more differentiated and noninvasive phenotype, as well as a high malignant, stem-cell like more invasive phenotype. Higher levels of GFAPδ are expressed in neurogenic stem-cell like cells of the healthy brain (Middeldorp & Hol, 2011; Roelofs et al., 2005; van den Berge et al., 2010) and in higher malignant astrocytoma (Andreiuolo et al., 2009; Brehar et al., 2014; Choi et al., 2009; Heo et al., 2012), and the GFAPδ/α ratio is increased in grade IV astrocytoma (Stassen et al., 2017). Therefore, cells with a high GFAPδ/α ratio might be the high malignant, stem-cell like more invasive cells of the GFAP cell population. These cells are present at lower numbers in low-grade astrocytoma and could potentially induce progression into higher malignancy grades. Differences in malignant behavior of cells with a high and low GFAPδ/α ratio support this hypothesis (Moeton et al., 2014; Stassen et al., 2017) and future studies should focus on unravelling the isoform-specific function in astrocytoma malignancy. In conclusion, information is lost when the expression of different GFAP isoforms is neglected and can be deceiving when GFAP is used to determine the differentiation state of a cell in experimental and clinical settings. Therefore, future studies need to focus on further identifying the GFAP positive cell population and make use of the possibility to discriminate between GFAP variants that could be fruitful to diagnosis and to the understanding of the molecular basis of glioma.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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